PI3K Isoform Dependence and Translational Regulation of c-MYC in PTEN-Deficient Leukemia

PI3K Isoform Dependence and Translational Regulation of c-MYC in PTEN-Deficient Leukemia

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ABSTRCT

T-cell acute lymphoblastic leukemia (T-ALL) is a lethal decrease that is usually caused by hyper-activation of PI3K signaling, generally featured with loss of *Pten*. In this thesis, using genetically engineered murine model of T-ALL initiated by *Pten*-loss, we studied the roles of different PI3K catalytic subunit isoforms in T cell specific PTEN null induced T-ALL. We show that neither p110α nor p110β isoform is critical for the maintenance of the T cell specific PTEN loss induced T-ALL, suggesting potential critical role of the PI3K p110δ and p110γ in PTEN-null induced T-ALL.

Highly elevated c-MYC oncoprotein abundance is found in most cases of T-cell acute lymphoblastic leukemia (T-ALL) and plays a key pathogenic role in this disease. Notably, the co-occurrence of c-MYC overexpression and PTEN-deficiency is common in T-cell acute lymphoblastic leukemia (T-ALL). However, the mechanistic link between the two events resulting in this correlation has remained unclear and unexploited therapeutically. Using the genetically engineered murine model of T-ALL initiated by *Pten*-loss and featuring spontaneous overexpression of c-MYC as well as a panel of PTEN-deficient c-MYC-high human T-ALL cell lines, we found that the abundance of c-

MYC in these cells is critically dependent on hyper-activation of PI3K-mTOR-S6K1 signaling. While multiple mechanisms have been proposed to allow PI3K signaling to regulate c-MYC expression, we found that the details of c-MYC regulation in T-ALL are unique, not occurring via the regulation of c-MYC stability by phosphorylation or the common translational initiation machinery via eIF4E. We demonstrate that the PI3K signaling pathway robustly regulates translation of *MYC* via eIF4A, a eukaryotic translation initiation factor with intrinsic RNA helicase activity. Notably, eIF4A inhibition, either by RNA interference or via the pharmacological inhibitor hippuristanol, efficiently suppresses *MYC* translation and tumor cell proliferation via a mechanism specifically dependent on the 5'UTR of c-MYC. Our study establishes a novel eIF4A-dependent mechanism of *MYC* translation essential in T-ALL, and identifies eIF4A as a promising therapeutic target in c-MYC-dependent hematological malignancies.

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TABLE OF CONTENTS

TABLE OF FIGURES

Chapter One

Introduction

1.1 T Cell Lymphoma and T cell acute lymphoblastic leukemia

1.1.1 T Cell Lymphoma

Lymphoma is the most common blood cancer type. There are two main types of lymphoma: the Hodgkin lymphoma and the non-Hodgkin lymphoma. Non-Hodgkin lymphoma accounts for more than 90% of all of the lymphoma cases. Lymphoma happens when cells of the immune system, called lymphocytes, grow out of control. There are two types of lymphocytes and thus two types of non-Hodgkin lymphomas, the B cell lymphoma and T cell lymphoma. T cell lymphoma usually originates in the thymus where the T cells develop and mature and then spread to peripheral lymphoid organs including the spleen and the lymph nodes (Hagenbeek and Spits, 2008). Typically, it appears as a solid tumor of transformed lymphoblasts, featured with enlarged nuclei. T cell lymphoma is less common than B cell lymphoma. It accounts for about 15% of non-Hodgkin lymphomas in the United States and it is more common in Asia. However, T cell lymphoma is a lethal disease and it has poor prognosis compared to B cell lymphoma (Tsukasaki et al., 2014). There is currently no targeted therapy available for T cell lymphoma. The commonly used therapy strategies include surgery, radiation, transplantation, and chemotherapy.

1.1.2 T Cell Acute Lymphoblastic Leukemia (T-ALL)

Since transformed T lymphocytes migrate via circulation, they usually cause leukemia featured with high white blood cell count and spread of lymphoblasts in the bone marrow. Due to the lymphoblastic origin and quick development of this type of leukemia, it is usually called as T cell acute lymphoblastic leukemia (T-ALL). T-ALL accounts for about 15% of pediatric cases and 20% of adults cases of acute leukemia (Ravandi et al., 2005). It is most often seen in children and adolescents. T-ALL is also a lethal disease. There is no targeted therapy for this disease. Commonly used therapies for T-ALL include chemotherapy, radiation and transplantation (Veigaard et al., 2014). Genetically, T-ALL is commonly featured with various genetic translocations due to the active recombination process of T cells during development. The mostly hyper-activated signaling pathways in T-ALL include the Notch signaling (accounts for about 50% of the T-ALL patients) (Pear and Aster, 2004) and the PI3K signaling (accounts for about 40% of the T-ALL patients) (Gutierrez et al., 2009).

1.2 PI3K Signaling and PI3K Catalytic Subunit Isoforms

1.2.1 PI3K Signaling

PI3K signaling pathway is one of the most frequently deregulated signaling pathways in various human cancers. As major effectors for extracellular stimuli, PI3Ks transduce signals by phosphorylating the 3’ position on phosphatidylinositol-4,5-bisphosphate

yielding phosphatidylinositol-3,4,5-trisphosphate (Engelman, 2009; Liu et al., 2009b). This process can be antagonized by the phosphatase PTEN, the main suppressor of PI3K signaling. Activation of PI3Ks will phosphorylate and thus activate the serine-threonine kinase AKT, the main downstream effector of PI3K, which will further activate multiple downstream pathways (Wong et al., 2010). The main downstream effecter of AKT is the mTOR complex1 (mTORC1), which in turn can activate its downstream S6 Kinase1 (S6K1) and phosphorylate the translation initiation factor 4E-binding protein1 (4EBP1), resulting in its dissociation from the translation initiation factor eIF4E and activation of cap-dependent translation initiation. Various downstream effectors of AKT can collectively promote cell growth, survival, and metabolism. Hyperactivation of PI3K signaling can stimulate multiple downstream signaling effects that collectively lead to oncogenesis (Engelman, 2009).

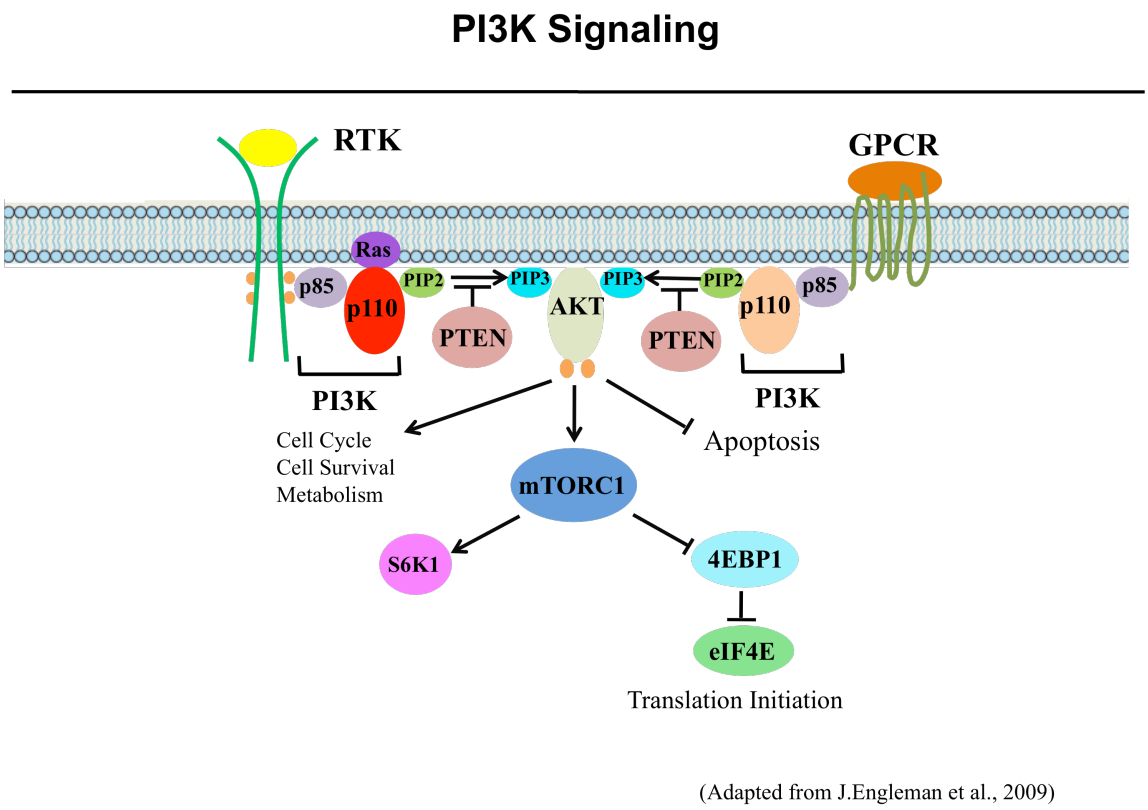
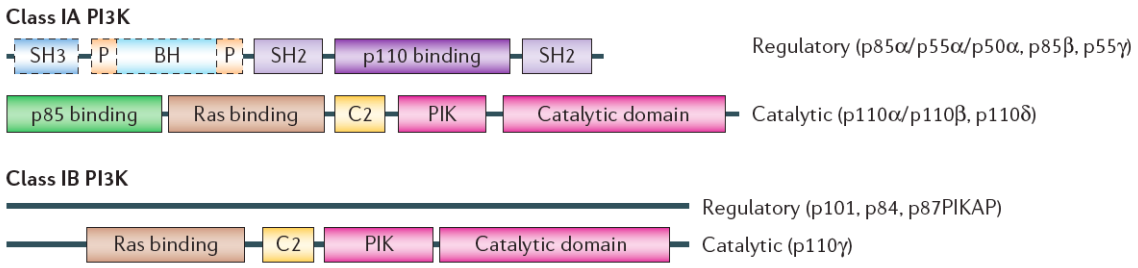


Figure 1.1 PI3K Signaling. PI3K Signaling is activated by receptor tyrosine kinases (RTKs), GPCR or Ras. Activation of PI3K leads to phosphorylation of PIP2 into PIP3, which further activates its downstream kinase AKT. AKT activation further activates multiple downstream effectors and the main one is the mTORC1 complex. mTORC1 phosphorylate S6K1 to activate its downstream signaling or phsophorylate 4EBP1 and thus activate translation initiation.

1.2.2 PI3K Catalytic Subunit Isoforms

There are three classes of PI3Ks of which the Class I PI3Ks are the most implicated class

in human cancers (Ilic and Roberts, 2010). Class I PI3Ks are responsible for the production of Phosphatidylinositol 3-phosphate (PI(3)P), Phosphatidylinositol (3,4)-bisphosphate (PI(3,4)P2), and Phosphatidylinositol (3,4,5)-trisphosphate (PI(3,4,5)P3). Class I PI3Ks are composed of a regulatory and a catalytic subunit. For Class IA PI3K, it is composed of a heterodimer of a p85 regulatory subunit and a p110 catalytic subunit. There are three variants of the p110 catalytic subunit named p110α, p110β, or p110δ catalytic subunit. The p110α and p110β subunits are expressed in all cells while p110δ is expressed primarily in leukocytes. The class IB PI3K is composed by the regulatory p101 and catalytic p110γ subunits (Liu et al., 2009a). It was long thought that different PI3K catalytic subunits have similar functions until recent studies suggest that the roles and involvement of different PI3K catalytic subunits in various models for tumorigenesis and maintenance. For example, in a prostate specific PTEN-Null driven cancer model, p110β, but not p110α was shown to be critical for tumor maintenance (Jia et al., 2008). In breast cancer driven by polyoma middle T antigen (MT) or HER2/Neu transgenic models, depletion of p110α blocked tumorigenesis. Surprisingly, p110β ablation resulted in the increase of tumorigenesis (Utermark et al., 2012). Another example is that p110α is responsible for the sustained survival of suprabasal cells of the epidermis in the absence of PTEN, p110β is important for the hyperproliferation of basal cells in PTEN hamartoma tumor syndrome (Wang et al., 2013). All these studies indicate the distinct roles of PI3K catalytic subunits in tumorigenesis under various genetic conditions and demonstrate the significance to study PI3K catalytic subunit isoform specificity for more effective and precise targeting of PI3Ks in human cancers.



(Klaus Okkenhaug et al., 2003)

Figure 1.2 PI3K Catalytic Subunit Isoforms. Class IA PI3K has three catalytic subunit isoforms, p110α, p110β, or p110δ. Class IB PI3K has one catalytic subunit isoform, p110γ.

1.3 PI3K Signaling in T-ALL

PI3K signaling has been reported to be frequently deregulated in T cell lymphoma and leukemia (Gutierrez et al., 2009). Mutation of PIK3CA and AKT; deletion and mutation of PTEN have been identified in more than 40% of human T cell lymphoma and leukemia samples (Gutierrez et al., 2009). Both T cell specific PTEN knockout mice and mice expressing constitutively activated AKT develop T cell lymphoma and leukemia with short latency (Hagenbeek and Spits, 2008; Kharas et al., 2010), strongly suggesting that the PTEN-PI3K-AKT axis can play a critical role in T cell lymphoma and leukemia. Also, multiple small molecule inhibitors of PI3K signaling have also been shown to be able to inhibit the proliferation of human T cell lymphoma/leukemia cell lines in vitro, indicating potential therapeutic effects of PI3K inhibitors for the treatment of T cell lymphoma/leukemia (Chiarini et al., 2009; Chiarini et al., 2010; Evangelisti et al., 2011). However, little is known about the isoform specificity of PI3K catalytic subunit isoforms

in T-ALL. It is unclear whether targeting specific PI3K isoform is enough to block T-ALL driven by PTEN-null deficient cancers and if yes, which isoform(s) would be. It is also unclear how PI3K signaling activates downstream effectors and thus cause oncogenesis in T-ALL.

1.4 PI3Ks Functions in T cells

As one of the signaling pathways critical for multiple cellular functions, various components of PI3K signaling have been reported to be important for the development of T cells. T cell specific knock-out mice models of AKT1/2 and PDK1 showed much smaller thymus compared to the wide type mice with blockage of T cell development from the DN3 to DN4 stage (Hinton et al., 2004; Juntilla et al., 2007). The functions of PI3K catalytic subunits in T cells also have been previously conducted, especially p110δ and p110γ, the two isoforms specifically expressed in leukocytes. Mice deficient in both p110γ and p110δ have a profound block in T cell development that occurs at the beta-selection checkpoint. pre-TCR-induced signaling is also greatly reduced in p110gamma-/-p110delta-/- thymocytes (Webb et al., 2005). Specifically, mice lacking expression of the PI3K p110γ subunit have a partial defect in T cell differentiation, which is restored after expression of an active PI3K mutant (Rodriguez-Borlado et al., 2003). p110γ -deficient T cells show reduced chemotactic responses to the lymphoid chemokines including CCL19, CCL21, and CXCL12 (Reif et al., 2004). p110γ is dispensable for constitutive migration of naive CD8 T cells and subsequent activation and differentiation into effector CD8 T cells, but plays a central role in the migration of effector CD8 T cells

into inflammatory sites (Martin et al., 2008). The Ras-p110γ interaction was also reported to be necessary for efficient β-selection-promoted proliferation of T cells (Janas and Turner, 2011). Dominant-activating germline mutations in p110δ result in T cell senescence and human immunodeficiency (Lucas et al., 2014). Antigen receptor signaling in T cells is impaired and immune responses in vivo are decreased in p110δ mutant mice (Okkenhaug et al., 2002). Specifically, p110δ mutant mice have attenuated function of CD4+CD25+Foxp3+ regulatory T cells (Tregs) featured with lower suppressor function in vitro and failed secretion of cytokines (Patton et al., 2006). p110δ has also been reported to promote Th17 differentiation in recent studies (Steinbach et al., 2014). On the other hand, the roles of p110α and p110β in normal T cell development and function are much less studied. Our unpublished data suggest that T cell specific p110α deletion caused in a much smaller thymus whereas p110β deletion is dispensable for T cell development.

1.5 c-MYC and Therapy for the c-MYC Oncoprotein

1.5.1 The c-MYC Oncoprotein

c-Myc is a basic-helix-loop-helix leucine zipper (bHLH-LZ) transcription factor that functions as a master regulator of the transcriptome (Lin et al., 2012b; Nie et al., 2012), thus governing many cellular processes, including cell proliferation and transformation. A wide spectrum of genomic and genetic alterations of MYC have been found in various tumors, including gene translocations, amplification, overexpression and occasional point

mutations (Beroukhim et al., 2010; Eilers and Eisenman, 2008; Meyer and Penn, 2008; Pomerantz et al., 2009; Setoodeh et al., 2013; Wright et al., 2010). In addition, increased abundance of the c-MYC protein is also frequently observed in cancer via distinct mechanisms such as alterations in upstream regulators or posttranslational modifications (Choi et al., 2010; Huang et al., 2004; Malempati et al., 2006; Wiegering et al., 2013). The upregulation of c-MYC occurs in a majority of human malignancies, and is usually associated with poor prognosis (Miyawaki et al., 2012; Pedersen et al., 2013; Ryan et al., 2012). Therefore, c-MYC represents a major target for human cancer therapy.

1.5.2 Targeted therapy for the c-MYC Oncoprotein

Despite decades of enormous effort to develop therapeutics to inhibit its function, direct targeting c-MYC via pharmacological agents has remained challenging, in part due to the fact that the c-MYC protein lacks a conventional druggable pocket (Darnell, 2002). Although it is known that the oncogenic activity of c-MYC requires dimerization with its binding partner MAX (Amati et al., 1993; Blackwood and Eisenman, 1991), efforts trying to inhibit such protein-protein interaction have also been unsuccessful (Nair and Burley, 2003). Therefore, c-MYC protein is long thought to be undruggable. Recent studies demonstrated the existence of synthetic lethal interactions between c-MYC activation and certain elements in a number of cellular pathways, such as protein sumorylation (Kessler et al., 2012), cell division (Horiuchi et al., 2012; Yang et al., 2010), and initiation of protein translation (Lin et al., 2012a), suggesting potential strategies to target c-MYC via inhibition of proteins critical for its function. In addition,

pharmaceutically targeting BET bromodomain proteins has been shown to effectively suppress MYC transcription, and consequently, the growth of some classes of c-MYC dependent tumors (Bandukwala et al., 2012; Cheng et al., 2013; Da Costa et al., 2013; Delmore et al., 2011; Henssen et al., 2013; Lockwood et al., 2012; Mertz et al., 2011; Ott et al., 2012; Shimamura et al., 2013; Tolani et al., 2013; Zuber et al., 2011). These findings have underscored the potential of indirectly targeting c-MYC through intervening in the molecular mechanisms that critical regulate c-MYC expression.

1.6 c-MYC in T-ALL

The Myc-TCRA translocation has been discovered in many human T cell lymphoma patients. Overexpression of the c-MYC protein is also frequent in various human T-ALL cell lines. Myc was identified as a critical downstream target of Notch1 signaling in T-ALL (Palomero et al., 2006). It was found to be widely elevated in several Pten null T cell lymphoma/leukemia mouse models (Guo et al., 2008; Liu et al., 2010; Zhang et al., 2011). Further investigations have verified the Myc-TCRA translocation and knock out of c-Myc in the hematopoietic system was able to significantly postpone T cell lymphomagenesis in Pten null mice (Zhang et al., 2011), suggesting that Myc may be critical for Pten null T cell lymphoma/leukemia. However, it is unclear whether this is purely due to additive oncogenic effects of the two lesions or to a more fundamental molecular connection between PI3K signaling and the c-Myc oncogene. If it is the latter, how these two oncogenic events are correlated and what would be the clinical relevance of the regulation is unknown.

1.7 c-MYC 5'UTR (5' Untranslatated Region) and RNA Helicases

The 5' UTR of c-MYC has an internal ribosome entry segment (IRES) featured with secondary structure (Stoneley et al., 1998). Cellular IRESes have been reported in multiple mRNAs critical for cell growth and proliferation. These genes usually function as oncogenes, such as CyclinD1 and c-Jun (Shi et al., 2005). Previous studies have suggested that the 5'UTR of c-MYC, similar to viral IRESes, may initiate translation in a cap-independent manner. Highly structured c-MYC 5'UTR can impede the binding and movement of the 40S ribosome. Therefore, the unwinding of c-MYC 5'UTR by RNA helicases are critical to expose the cellular IRES and thus initiate translation (Carter et al., 1999). On the other hand, it is unknown how the 5'UTR of c-MYC is regulated by upstream signaling and what is (are) the RNA helicase(s) that can unwire c-MYC 5'UTR and thus initiate translation. Multiple RNA helicases have been studied in various systems. The mostly reported RNA helicase is eukaryotic translation initiation factor eIF4A (also named as DDX2) (Parsyan et al., 2011). Together with its accessory proteins including eIF4B and eIF4H, eIF4A is thought to unwind structured mRNAs. Growing recent evidence also suggested that other DEAD-BOX RNA helicases such as DHX9, DHX29, are also important for translation initiation and may promote the scanning processivity of the 40S subunit. They either function alone or can synergize with eIF4A to facilitate translation initiation of certain mRNAs (Parsyan et al., 2011). So far, there is lack of ribosome profiling studies to identify targets of eIF4A. Given that c-MYC 5'UTR is highly structured, it is likely that c-MYC is one of the targets of eIF4A.

Summary

Both the PI3K signaling and the c-MYC oncogene have been identified as critical lesions in T-ALL, demonstrating the promising potential to targets these two oncogenic nodes for efficient targeted therapy in human cancers. Given the fact that T-ALL is lethal and currently no targeted therapy is available for T-ALL, there is urgent need to understand how PI3K signaling drives oncogenesis in T-ALL for more effective targeting of the signaling and also to test whether there would be isoform specificity of the enzyme and thus been utilized to design isoform specific inhibitors. In addition, as one of the mostly hyper-activated oncogenes in various human cancers, c-MYC presents a promising target for effective therapy in more than 70% of human cancers dependent on c-MYC. However, due to the difficulty to target transcription factors, c-MYC has long thought to be undruggable until recent discoveries of bromo domain inhibitors. Given the fact that c-MYC is spontaneously widely elevated in PTEN null driven T-ALL, it is intriguing to hypothesize that there could be clinically relevant regulatory mechanisms of c-MYC by the PI3K signaling pathway and thus could be utilized for novel strategy to target c-MYC dependent human cancers. In this thesis, we are mainly interested to address two questions. First, whether there is any isoform specificity of PI3K catalytic subunit isoforms and if yes, what isoform(s) is (are) critical for PTEN null driven T-ALL and second, whether there is any mechanistic correlation between the PI3K signaling and c-MYC oncogene. If yes, whether it could be utilized to discover novel strategy to target the currently undruggable c-MYC oncogene is unclear.

CHAPTER TWO

PI3K Catalytic Subunit Isoform Specificity in PTEN-deficient T-ALL

Introduction

As one of the most frequently hyperactivated signaling pathways in various human cancers, PI3K signaling presents a very intriguing signaling pathway for targeted cancer therapy (Liu et al., 2009b). The PI3K is composed of a regulatory p85 subunit and a catalytic p110 subunit. There are different catalytic subunit isoforms of PI3Ks in various human cancers, including the p110α, p110β, p110δ and p110γ isoforms (Fritsch and Downward, 2013), each is capable to regulate distinct biological functions in cell signaling and tumorigenesis. Recent interest has drawn to study the function of isoform specificity for more effective targeting of the PI3K signaling in human cancers. Given our previous studies in other cancer systems demonstrating the critical roles of the p110α or p110β isoforms in tumorigenesis and tumor maintenance (Utermark et al., 2012; Wang et al., 2013), we used a T cell specific PTEN null mice model (Lck+ Pten-/-) to check whether the p110α and/or p110β isoforms is critical for PTEN null induced T-ALL.

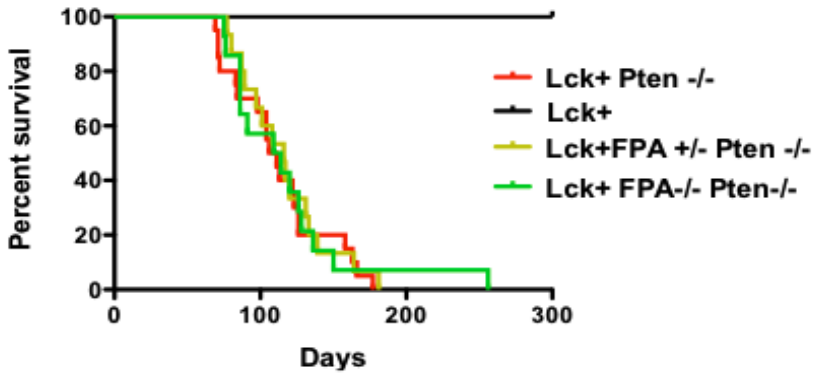
Contributions: Dr. Thomas Roberts and I designed the project and experiments together. I performed most of the experiments described in this chapter. Other peoples' contribution is stated in the thesis.

Results

p110α Loss Fails to Block T Cell Lymphoma Driven By Pten Loss

To start, we first tested whether knocking out the PI3K p110α isoform in PTEN null leukemia can block tumorigenesis, since p110α is the isoform mostly reported to be critical for PI3K signaling during tumorigenesis. FPA f/+ or FPA f/f mice were crossed to Lck+ Pten-/- mice to generate one allele or two alleles deletion of PIK3CA (Lck+ FPA+/- Pten-/- mice and Lck+ FPA-/- Pten-/- mice). Interestingly, deletion of neither one allele nor both alleles of PIK3CA did not postpone oncogenesis latency or decrease penetration rate of Lck+ Pten-/- mice (Fig 2.1), suggesting that p110α may not be critical for the maintenance of PTEN-null T-ALL.

A



B

Genotype	Number	Latency	Penetration
Lck+ PTEN-/-	20	112	100%
Lck+ FPA+/- PTEN-/-	15	116	100%
Lck+ FPA-/- PTEN-/-	14	117	100%

Figure 2.1 p110α loss fails to block T cell lymphoma driven by Pten loss

(A) Kaplan-Meier survival curve for Lck+ PTEN-/- mice (red), Lck+ FPA +/- PTEN-/- (yellow) mice, Lck+ FPA-/- PTEN-/- (green) and control Lck+ mice (black).

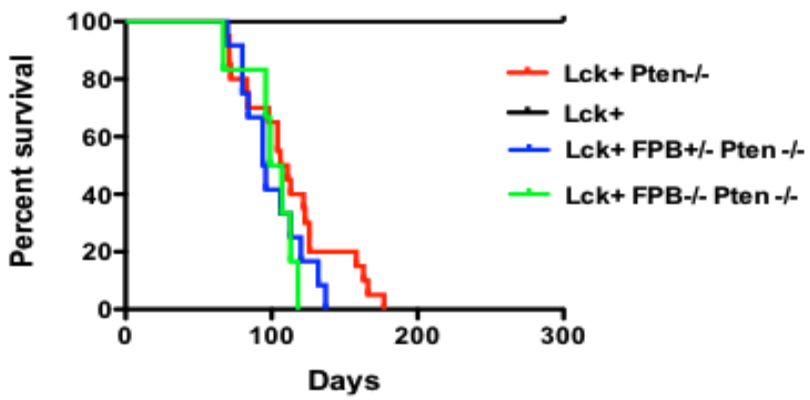
(B) Summary of survival conditions for Lck+PTEN-/-, Lck+ FPA+/- PTEN-/- and Lck+ FPA-/- PTEN-/- mice.

Contributions: I performed all the experiments for this figure.

p110β Loss Fails to Block T Cell Lymphoma Driven By Pten Loss

We then tested whether p110β can block tumorigenesis in Pten null driven T-ALL. FPB f/+ or FPB f/f mice were crossed to Lck+ Pten-/- mice to generate one allele or two alleles deletion of PIK3CB (Lck+ FPB+/- Pten-/- mice and Lck+ FPB-/- Pten-/- mice). To our surprise, deletion of neither one allele nor both alleles of PIK3CB also did not of Lck+ Pten-/- mice (Fig 2.2), suggesting that p110β may also not be critical for the maintenance of PTEN-null T-ALL.

A



B

Genotype	Number	Latency	Penetration
Lck+PTEN-/-	20	112	100%
Lck+ FPB+/- PTEN-/-	12	101	100%
Lck+ FPB-/- PTEN-/-	9	120	100%

Figure 2.2 p110β loss fails to block T cell lymphoma driven by Pten loss

(A) Kaplan-Meier survival curve for Lck+ PTEN-/- mice (red), Lck+ FPB +/- PTEN-/- (yellow) mice, Lck+ FPB-/- PTEN-/- (green) and control Lck+ mice (black).

(B) Summary of survival conditions for Lck+PTEN-/- mice, Lck+ FPB+/- PTEN-/- mice and Lck+ FPB-/- PTEN-/- mice.

Contributions: I performed all the experiments in this figure.

p110α or p110β Deleted Pten Null Mice Have Similar Phenotype as Pten Null Mice

To test whether p110α or p110β deletion may change the phenotype of Pten null driven T-ALL, we first measured the organ weight of moribund mice from various genetic backgrounds. Consistent with survival data, the organ weights of different genetic backgrounds don't show statistic difference (Fig 2.3). We also confirmed similar phenotype via HE staining demonstrating the invasion of lymphblasts invading into various organs of the immune system (Fig 2.4).

To further confirm similar phenotype of mice from different background, we confirmed the elevation of white blood cell counts in various genetic backgrounds (Fig 2.5). They also showed expansion and invasion of DP (CD4+CD8+) cells or SP4 (CD4+CD8-) cells in the thymus, lymph nodes, bone marrow and spleen (Fig 2.6A), with highly increased percentage of activated T cells (CD5+ TCR+) and loss of B cells (CD19+) in the lymph nodes, bone marrow and spleen of moribund mice. All these data collectively demonstrate that the T-ALL developed in Lck+ FPB+/- PTEN-/- mice and Lck+ FPB-/- PTEN-/- mice is similar to Lck+ PTEN-/- mice, suggesting that neither p110α nor p110β is critical for Pten null induced T-ALL.

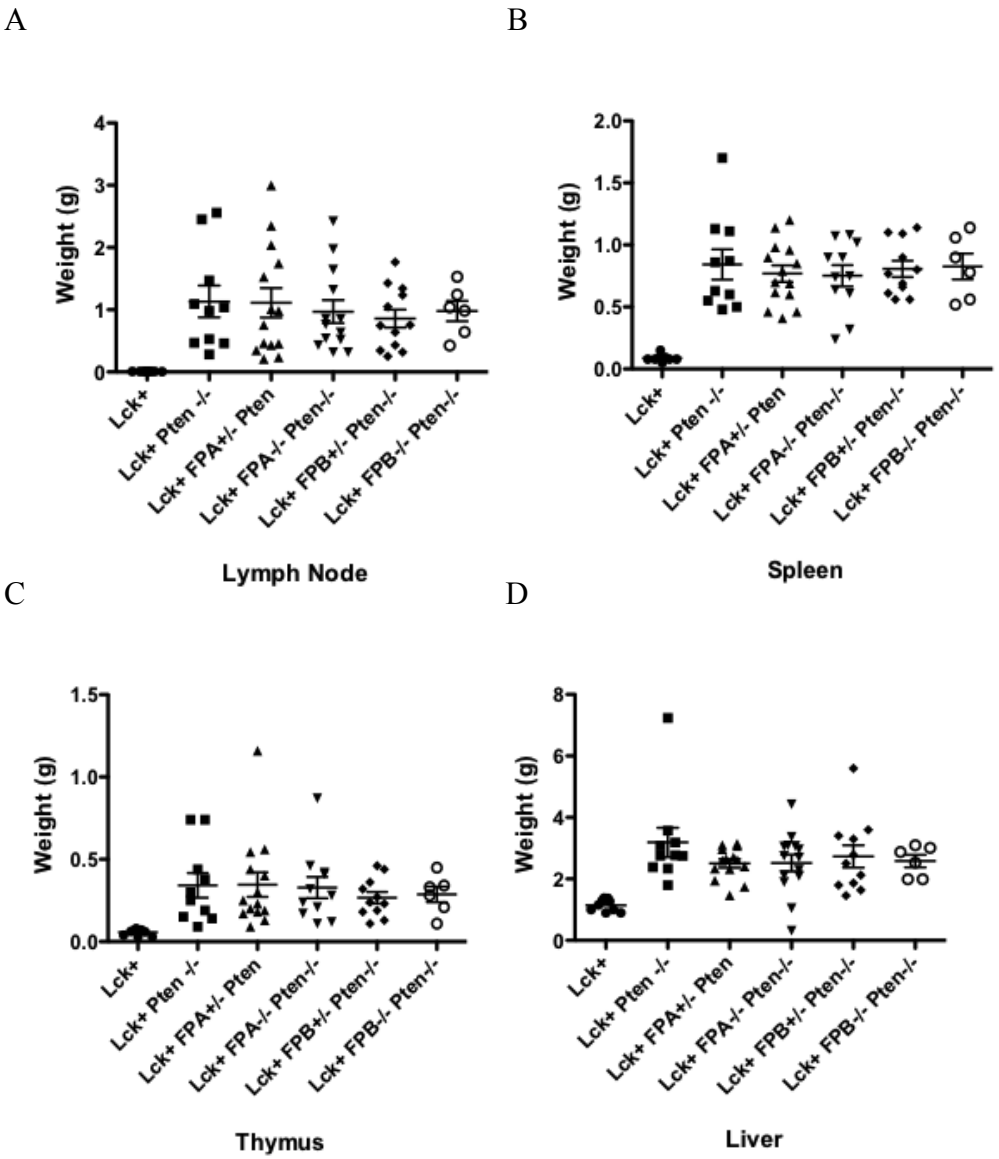


Figure 2.3 p110α and p110β deleted Pten null mice also have enlarged organs

Lymph node **(A)**, Spleen **(B)**, Thymus **(C)** and Liver **(D)** weight of control Lck+ mice, Lck+ PTEN-/- mice, Lck+ FPA +/- PTEN-/- mice, Lck+ FPA-/- PTEN-/- mice, Lck+ FPB+/- PTEN-/- mice and Lck+FPB-/- PTEN-/- mice.

Contributions: I performed all the experiments for this figure.

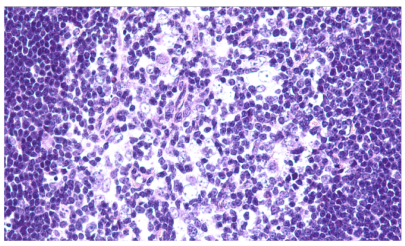
Figure 2.4 Lck+ Pten-/- FPA-/- mice and Lck+ Pten-/- FPB-/- mice showed similar HE staining phenotype compared to Lck+ Pten-/- mice.

Representative H&E-stained primary thymus **(A)**, lymph nodes **(B)**, and spleen sections **(C)** and representative Wright-giemsa stained primary bone marrow cytospin sections **(D)** from moribund Lck+ Pten-/- mice, Lck+ Pten-/- FPA-/- mice, Lck+ Pten-/- FPB-/- mice and age controlled Lck- Ptenf/f mice.

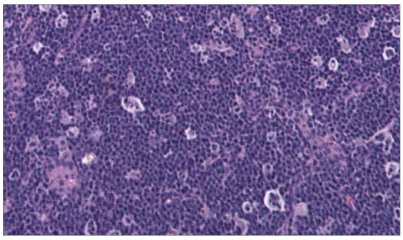
Contributions: I performed all the experiments for this figure.

Figure 2.4 (Continued)

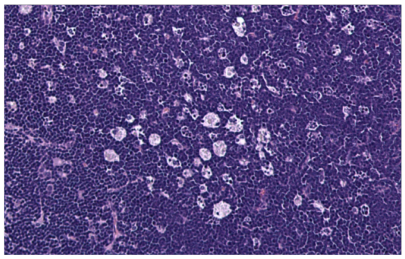
A



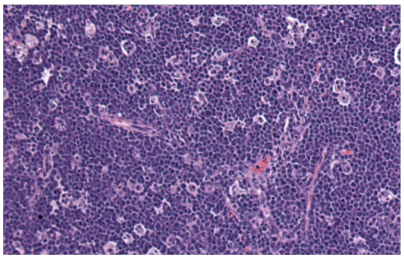
Lck- Pten f/f



Lck+ Pten -/-

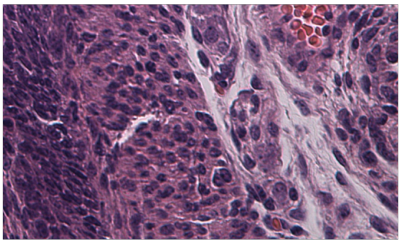


Lck+ Pten -/- FPA -/-

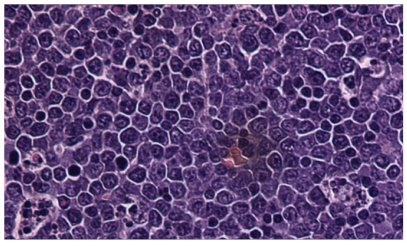


Lck+ Pten -/- FPB -/-

B



Lck- Pten f/f



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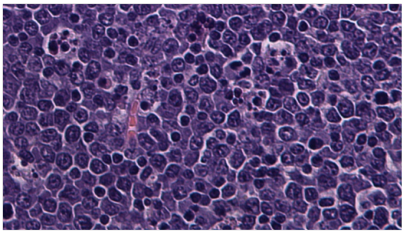
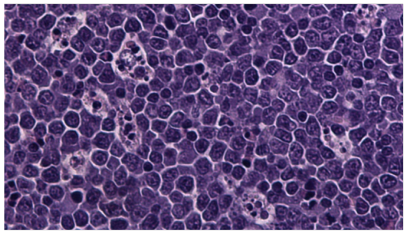


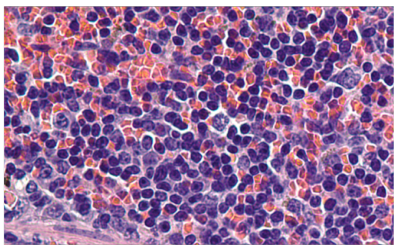
Lck+ Pten -/- FPA -/-



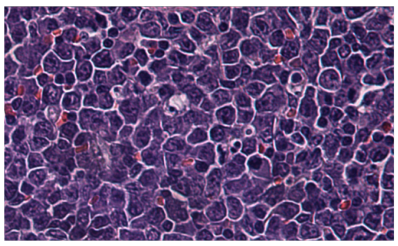
Lck+ Pten -/- FPB -/-

Figure 2.4 (Continued)

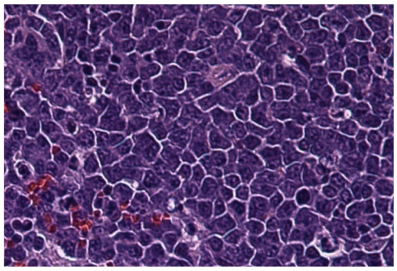
C



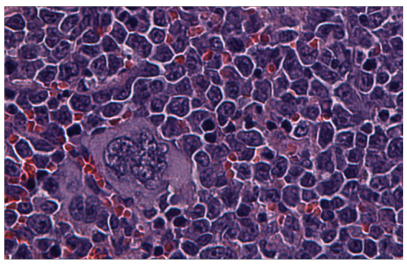
Lck- Pten f/f



Lck+ Pten -/-

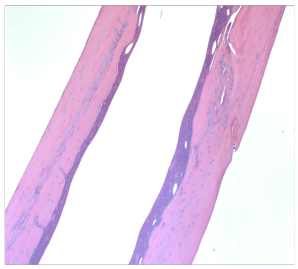


Lck+ Pten -/- FPA -/-

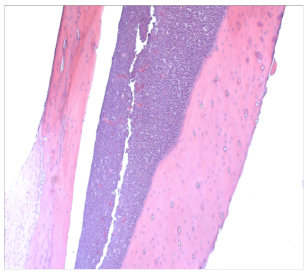


Lck+ Pten -/- FPB -/-

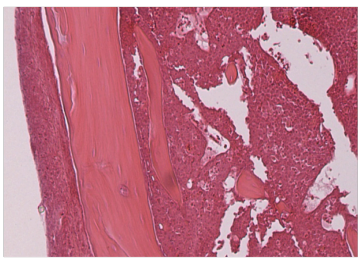
D



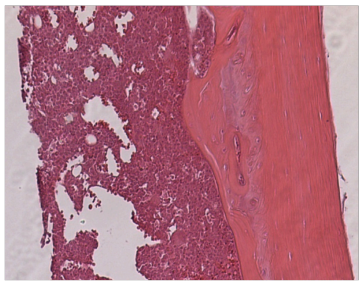
Lck- Pten f/f



Lck+ Pten -/-



Lck+ Pten -/- FPA -/-



Lck+ Pten -/- FPB -/-

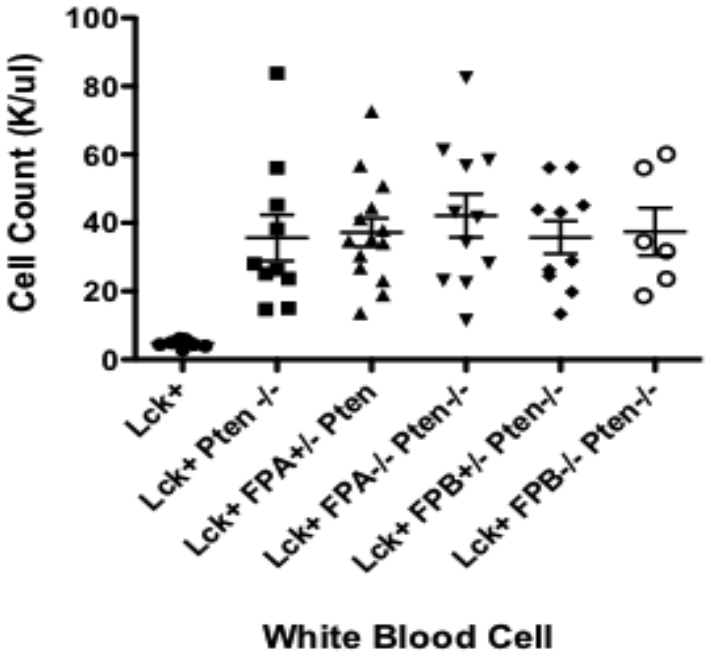


Figure 2.5 Lck+ Pten-/- FPA-/- mice and Lck+ Pten-/- FPB-/- mice showed similar white blood cell counts compared to Lck+ Pten-/- mice.

Contributions: I performed all the experiments in this figure.

Figure 2.6 Lck+ Pten-/- FPA-/- mice and Lck+ Pten-/- FPB-/- mice showed similar FACS staining pattern compared to Lck+ Pten-/- mice.

Expansion and invasion of DP or SP4 cells **(A)**, CD5+ activated T cells **(B)** and loss of CD19+ B cells **(C)** in the thymus, lymph nodes, spleen and bone marrow of Lck+ Pten-/- FPA-/- mice, Lck+ Pten-/- FPB-/- mice and Lck+ Pten-/- mice.

Contributions: I performed all the experiments for this figure.

Figure 2.6 (Continued)

A

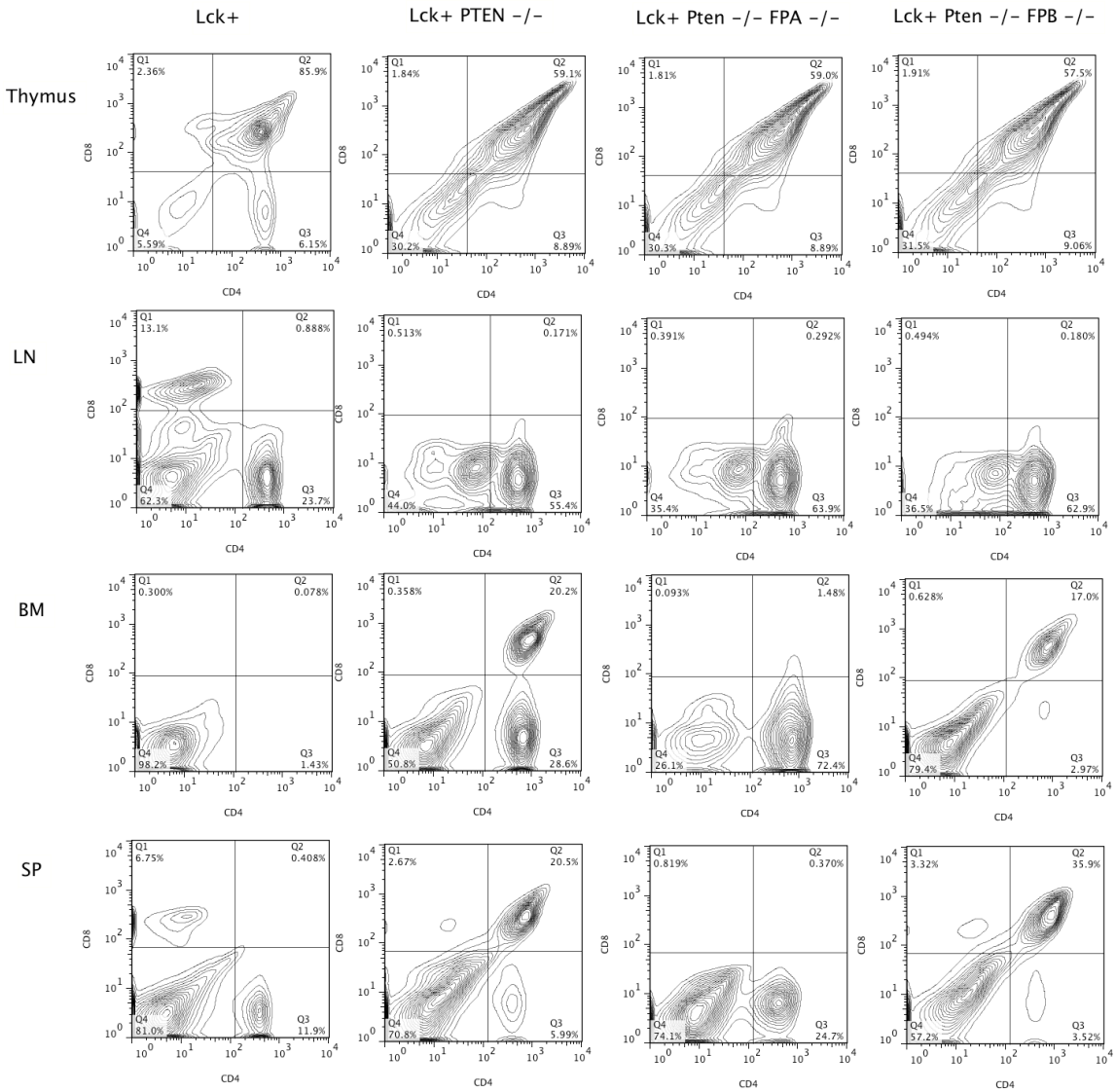


Figure 2.6 (Continued)

B

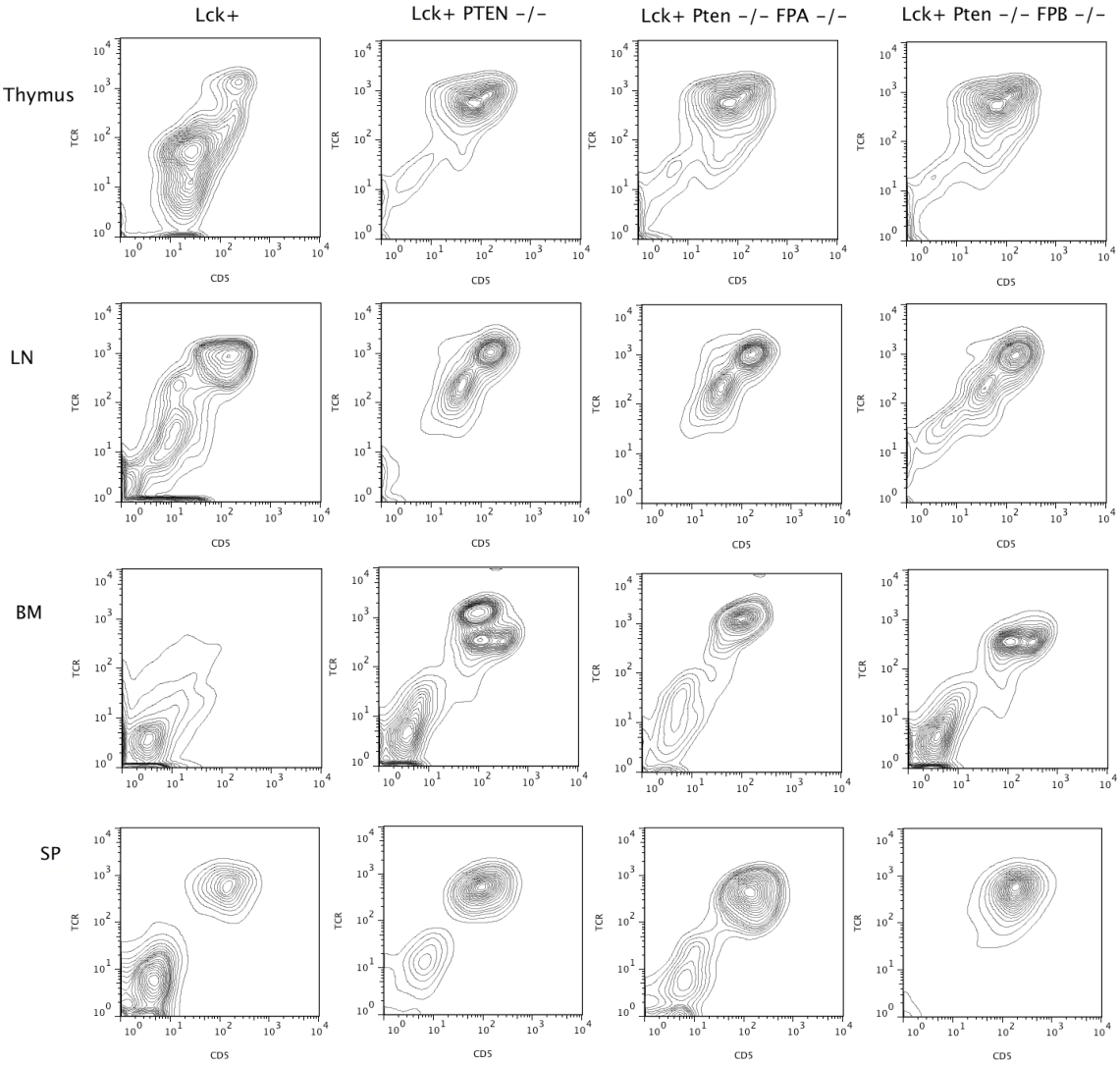
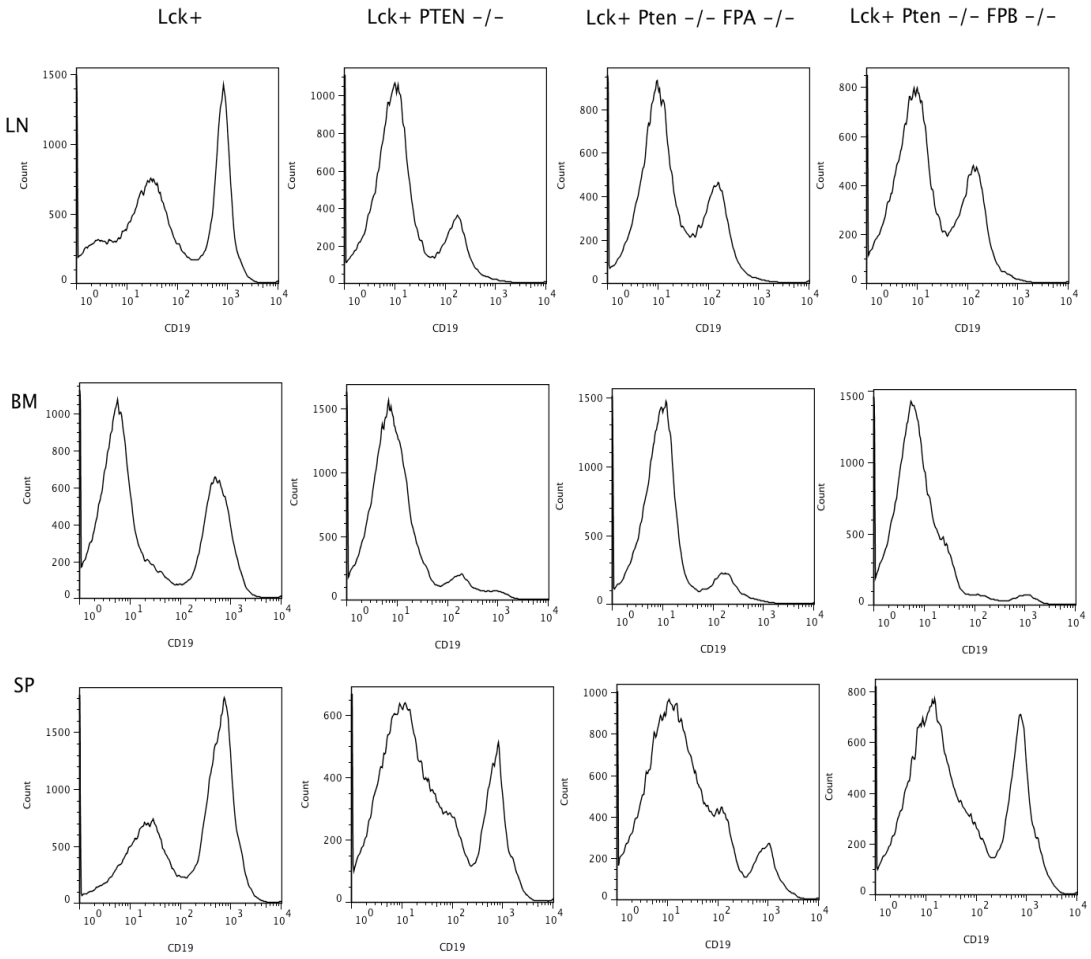


Figure 2.6 (Continued)

C



Contributions: I performed all the experiments for this figure.

Discussion and Future Directions

It was a surprise that neither p110α nor p110β affects tumorigenesis and tumor maintenance of PTEN null induced T-ALL given the fact that either or both of them are critical for PTEN null tumors in other systems. As we were doing the study, one paper published in 2012 in the journal of Cancer Cell stating that non-classical isoforms of PI3K, including the p110δ and p110γ, are critical for the maintenance of PTEN null T-ALL. In this study, they also used Lck+ PTEN-/- mice as the model system and showed that deletion of both p110δ and p110γ block tumorigenesis (Figure 2.7). This study also used a small molecule inhibitor CAL-130, a dual inhibitor of p110δ and p110γ, and showed effective inhibition of tumor growth *in vivo*, indicating the efficiency of blocking p110δ and p110γ to inhibit PTEN null induced T-ALL (Figure 2.8).

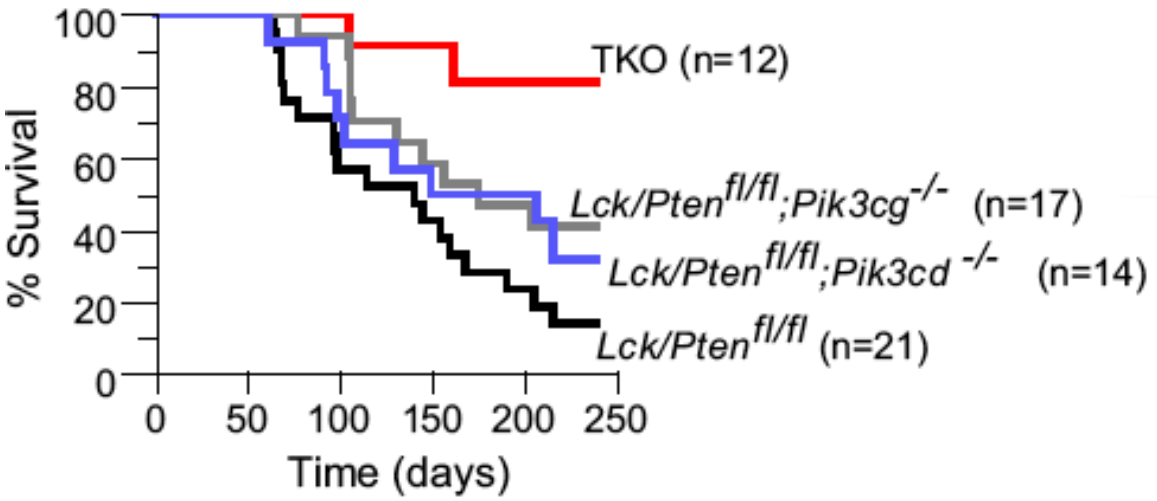


Figure 2.7 Kaplan-Meier survival curve for Lck+ PTEN-/- mice (black), Lck+ FPD-/- PTEN-/- (blue) mice, Lck+ FPG-/- PTEN-/- (grey), and triple knock out (TKO) mice. (P. Subramaniam et al., 2012)

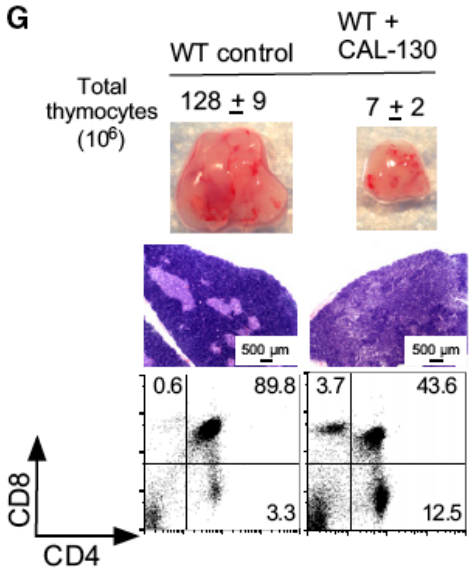


Figure 2.8 Phenotypic analyses of thymus from mice treated with either CAL-130 (10 mg kg^{-1} every 8 hr) or vehicle control for 7 days. (P. Subramaniam et al., 2012)

It is very interesting that p110δ and p110γ are critical for PTEN null driven T-ALL, since it is different from other PTEN null driven cancers commonly featured with critical roles of p110α and p110β. One potential explanation is that p110δ and p110γ are mostly expressed in leucocytes, including B cells and T cells. The results from our study and others indicate that in the absence of PTEN-mediated regulation, distinct class I PI3K can predominate in the development and survival of tumors in a manner that is most likely to involve isoforms that normally play a critical role in the function of that particular cell type. Given the possibility of potential toxicities that would be associated with pan-PI3K inhibitor or AKT inhibitors, the inhibition of p110δ and p110γ would be more specific.

Recent studies have also shown the existence of a complex signaling system involving PI3K signaling between the leukemic cells and stroma cells in the tumor microenvironment (Ayala et al., 2009; Konopleva et al., 2009; Burger et al., 2009). This is also supported by the recent studies that the PI3K p110δ inhibitor CAL-101 reduces levels of circulating chemokines known to be critical for tissue localization of chronic lymphocytic leukemic (CLL) cells (Hoellenriegel et al., 2011). Therefore, the study of PI3K isoforms may also contribute to the study of micro-environmental interactions via the PI3K signaling in T-ALL as well.

Experimental Procedures

Mice

Lck+ (Hennet et al., 1995) and PTEN f/f (Lesche et al., 2002) mice were crossed to generate Lck+ PTEN f/f mice for this study. PIK3CA f/f mice, PIK3CA f/+ mice, PIK3CB f/f mice and PIK3CB f/+ mice were crossed with Lck+ PTEN f/f to generate various genetic background mice for this study. All animals were housed and treated in accordance with protocols approved by the Institutional Animal Care and Use Committees at Dana-Farber Cancer Institute and Harvard Medical School.

FACS Analysis

Cells were isolated and stained with anti-CD4, anti-CD8, anti-TCR, anti-CD5 and anti-CD19 antibodies (BD Biosciences) according to the manufacture's instructions. Briefly, cells were washed with PBS, blocked with 3% BSA and then stained with antibodies for one hour. Cells were then washed again and analyzed for surface marker staining using BD FACSAria IIu followed by Flowjo analysis.

Histology, Wright-giemsa Staining and White Blood Cell Count

For histology analysis, primary tissues were isolated, fixed with 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin by the Dana

Farber/Harvard Cancer Center Rodent Histopathology Core. For Wright-giemsa staining, cytospin slides (Thermo Scientific) with primary mouse bone marrow cells and peripheral blood smear slides were stained according to routine method (Lillie, 1944) using Wright-giemsa stain (Electron Microscopy Sciences). Mouse peripheral white blood cell counts were measured using HEMAVET Multispecies Hematology Analyzer (Drew Scientific).

CHAPTER THREE

Translational Regulation of c-MYC in PTEN-deficient Leukemia

eIF4A is Essential for the Translation of c-MYC in PTEN-Deficient Leukemia

Authors

Haoxuan Tong, Alfredo Csibi, Yubao Wang, Lukas Baitsch, Thanh Von, Haluk Yuzugullu, Qi Wang, Jon Aster, Gerhard Wagner, Thomas M. Roberts, John Blenis, Jean J. Zhao

This chapter is adapted from a manuscript been submitted.

Contributions: Dr. Thomas Roberts, Dr. Jean Zhao, Dr. John Blenis and I designed the project and experiments together. I performed most of the experiments described in this manuscript. Other peoples' contribution is stated in the thesis. Dr. Jean Zhao, Dr. Thomas Roberts, Dr. John Blenis and I wrote the manuscript together.

Abstract

Highly elevated c-MYC oncoprotein abundance is found in most cases of T-cell acute lymphoblastic leukemia (T-ALL) and plays a key pathogenic role in this disease. Notably the co-occurrence of c-MYC overexpression and PTEN-deficiency is common in T-cell acute lymphoblastic leukemia (T-ALL). However, the mechanistic link between the two events resulting in this correlation has remained unclear and unexploited therapeutically. Using a genetically engineered murine model of T-ALL initiated by *Pten*-loss and

featuring spontaneous overexpression of c-MYC as well as a panel of PTEN-deficient MYC-high human T-ALL cell lines, we found that the abundance of c-MYC in these cells is critically dependent on hyper-activation of PI3K-mTOR-S6K1 signaling. While multiple mechanisms have been proposed to allow PI3K signaling to regulate c-MYC expression, we found that the details of MYC regulation in T-ALL are unique, not occurring via the regulation of MYC stability by phosphorylation or the common translational initiation machinery via eIF4E. We demonstrate that the PI3K signaling pathway robustly regulates translation of *MYC* via eIF4A, a eukaryotic translation initiation factor with intrinsic RNA helicase activity. Notably, eIF4A inhibition, either by RNA interference or via the pharmacological inhibitor hippuristanol, efficiently suppresses *MYC* translation and tumor cell proliferation via a mechanism specifically dependent on the 5'UTR of c-MYC. Our study establishes a novel eIF4A-dependent mechanism of *MYC* translation essential in TALL, and identifies eIF4A as a promising therapeutic target in c-MYC-dependent hematological malignancies.

Results

To establish a T-ALL tumor model driven by *Pten* loss, we crossed floxed *Pten* mice (Lesche et al., 2002) with transgenic mice in which *Cre* expression is driven by the proximal *Lck* promoter (*LckCre*) to effect genetic ablation of *Pten* specifically in T lymphocytes (Hennet et al., 1995). Consistent with previous studies (Hagenbeek and Spits, 2008), most $LckCre^{+}/Pten^{f/f}$ mice developed T cell malignancies (latency, 120 days; penetration, 93%) (Figure 3.1A). These mice displayed significant size increases in the

spleen, liver, thymus, and lymph nodes (Figure 3.1B) largely due to invasion of lymphoblasts (Figure 3.1C). White blood cell counts were also highly elevated in the peripheral blood of moribund *LckCre*$^{+}$*/Pten*$^{f/f}$ mice (Figure 3.1D), indicating the development of leukemia. Similar to previously reported *VecCre*$^{+}$*/Pten*$^{f/f}$, *Mx1Cre*$^{+}$*/Pten*$^{f/f}$ and *CD4Cre*$^{+}$*/Pten*$^{f/f}$ mice (Guo et al., 2008; Liu et al., 2010; Zhang et al., 2011), c-Myc protein levels in the thymocytes of moribund *LckCre*$^{+}$*/Pten*$^{f/f}$ mice were dramatically elevated (Fig 3.2A).

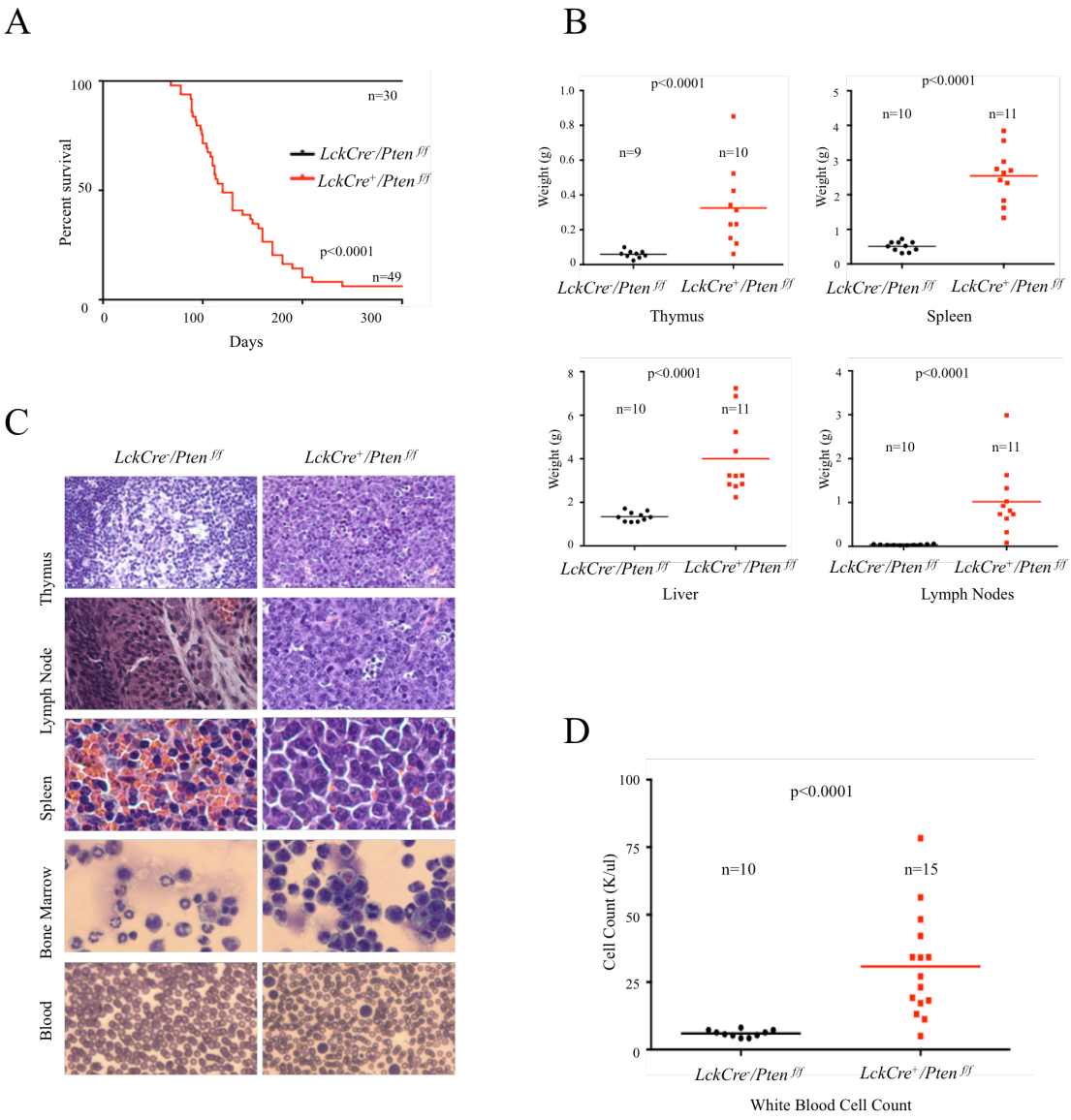


Figure 3.1 *LckCre+/Pten$^{f/f}$* mice developed T-ALL.

(A) Kaplan-Meier survival curve for *LckCre^{+}/Pten$^{f/f}$* mice (red) (*n*=49, median survival 120d), and *LckCre^{-}/Pten$^{f/f}$* control mice (black) (*n*=30).

Figure 3.1 (Continued)

(B) Tissue weights of thymus, spleen, liver and lymph nodes of *LckCre*$^{+}$*/Pten*$^{f/f}$ moribund mice (red) is significantly higher than that of age matched *LckCre*$^{-}$*/Pten*$^{f/f}$ control mice (black).

(C) Representative H&E-stained primary thymus, lymph nodes, and spleen sections and representative Wright-giemsa stained primary bone marrow cytospin sections and pheripheral blood smear sections from *LckCre*$^{+}$*/Pten*$^{f/f}$ moribund mice and age matched *LckCre*$^{-}$*/Pten*$^{f/f}$ control mice.

(D) White blood cell counts of peripheral blood from *LckCre*$^{+}$*/Pten*$^{f/f}$ moribund mice (red) is significantly higher than that of age matched *LckCre*$^{-}$*/Pten*$^{f/f}$ control mice (black).

Contributions: Thanh Von assisted with mice handling in this figure. Dr. Roderick Bronson and the Dana-Farber/Harvard Cancer Center Rodent Histopathology Core conducted histopathological analyses for this figure. I performed the rest of the experiments for this figure.

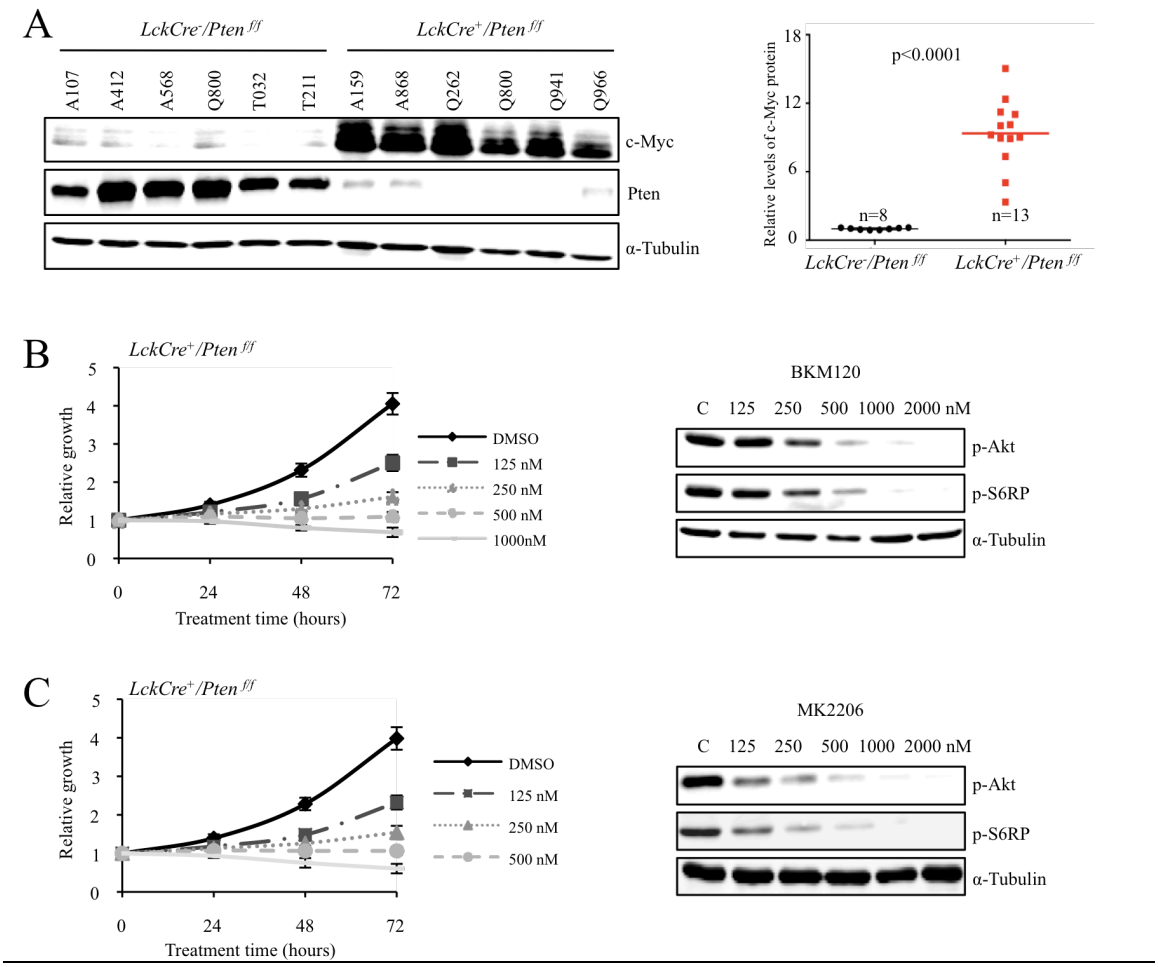


Figure 3.2 *Pten*-Null Driven T-ALL with Over-expressed c-Myc is Sensitive to PI3K/AKT Inhibition

(A) The abundance of c-Myc protein in the thymocytes of *LckCre*$^{+}$*/Pten*$^{f/f}$ moribund mice is significantly higher than that of age matched *LckCre*$^{-}$*/Pten*$^{f/f}$ healthy mice.

Figure 3.2 (Continued)

(B-C) PI3K or Akt inhibition by BKM120 or MK2206 treatment as indicated reduced both the growth and p-Akt in *LckCre*$^{+}$*/Pten*$^{f/f}$ primary tumor cells. For growth inhibition, indicated drugs were applied at indicated concentrations for indicated hours before measurement; for Western blotting, cells were treated with indicated drugs for one hour. DMSO was used as negative control.

Contributions: I performed all the experiments for this figure.

Our previous work in breast cancer models demonstrated that c-Myc overexpression causes resistance to PI3K inhibition (Ilic et al., 2011; Liu et al., 2011). To test whether leukemic cells with overexpressed c-Myc from *LckCre*$^{+}$*/Pten*$^{f/f}$ mice are also resistant to PI3K inhibition, we treated primary tumor cells with BKM120 (Elfiky et al., 2011), a pan-PI3K inhibitor currently under clinical evaluation. To our surprise, these primary T-ALL cells with robust c-Myc overexpression were highly sensitive to PI3K inhibition (Fig 3.2B). Similar results were observed in these cells when treated with MK2206, an AKT inhibitor currently in clinical trials (Chandarlapaty et al., 2011) (Fig 3.2C). These data indicate that, despite its markedly overexpressed c-Myc, T-ALL driven by *Pten*-loss in this murine model is still dependent on the PI3K-Akt pathway.

To determine whether c-Myc levels in *Pten*-null driven T-ALL are altered in response to PI3K or Akt inhibition, we treated primary tumor cells isolated from moribund

LckCre^{+}*/Pten*$^{f/f}$ mice with either BKM120 or MK2206, and then measured the protein abundance of c-Myc. Notably, both PI3K and AKT inhibitors dramatically decreased the abundance of c-Myc protein in these cells in a dose-dependent manner (Fig 3.3A-3.3B). Furthermore, we found that restoring the expression of Pten in these primary *Pten*-null T-ALL cells greatly reduced the abundance of c-Myc protein as well as the phosphorylation of both Akt and S6RP (Fig 3.3C). These data indicate that the PI3K/Akt signaling pathway tightly controls the abundance of c-Myc protein in this T-ALL model driven by *Pten*-loss.

We next extended our finding to human T cell lymphoma/leukemia cell lines. We screened a panel of T cell lymphoma or T-ALL cells. Out of thirteen cell lines tested, we found that six cell lines demonstrated either low or undetectable expression of PTEN (Fig 3. 3D). Notably, in all the six cell lines, AKT inhibition by MK2206 decreased c-MYC protein levels (Fig 3.3E). Ectopic expression of PTEN also decreased c-MYC levels in human PTEN-deficient T-ALL cell lines (Fig 3. 3F). These results suggest the link between PI3K/AKT pathway activation and c-MYC protein elevation observed in our murine model of *Pten*-null T-ALL is also evident in most PTEN-deficient human T-ALL cell lines.

Figure 3.3 Inhibition of PI3K or AKT Activity Reduces the Amount of c-MYC Protein in both Murine *Pten*-Null T-ALL and Human PTEN-Deficient T-ALL

(A-B) The abundance c-Myc protein in *LckCre*$^{+}$*/Pten*$^{f/f}$ primary tumor cells was reduced upon one hour treatment with BKM120 or MK2206 in a dose-dependent manner.

(C) Ectopic expression of Pten suppressed c-Myc protein level and PI3K signaling in *LckCre*$^{+}$*/Pten*$^{f/f}$ primary tumor cells.

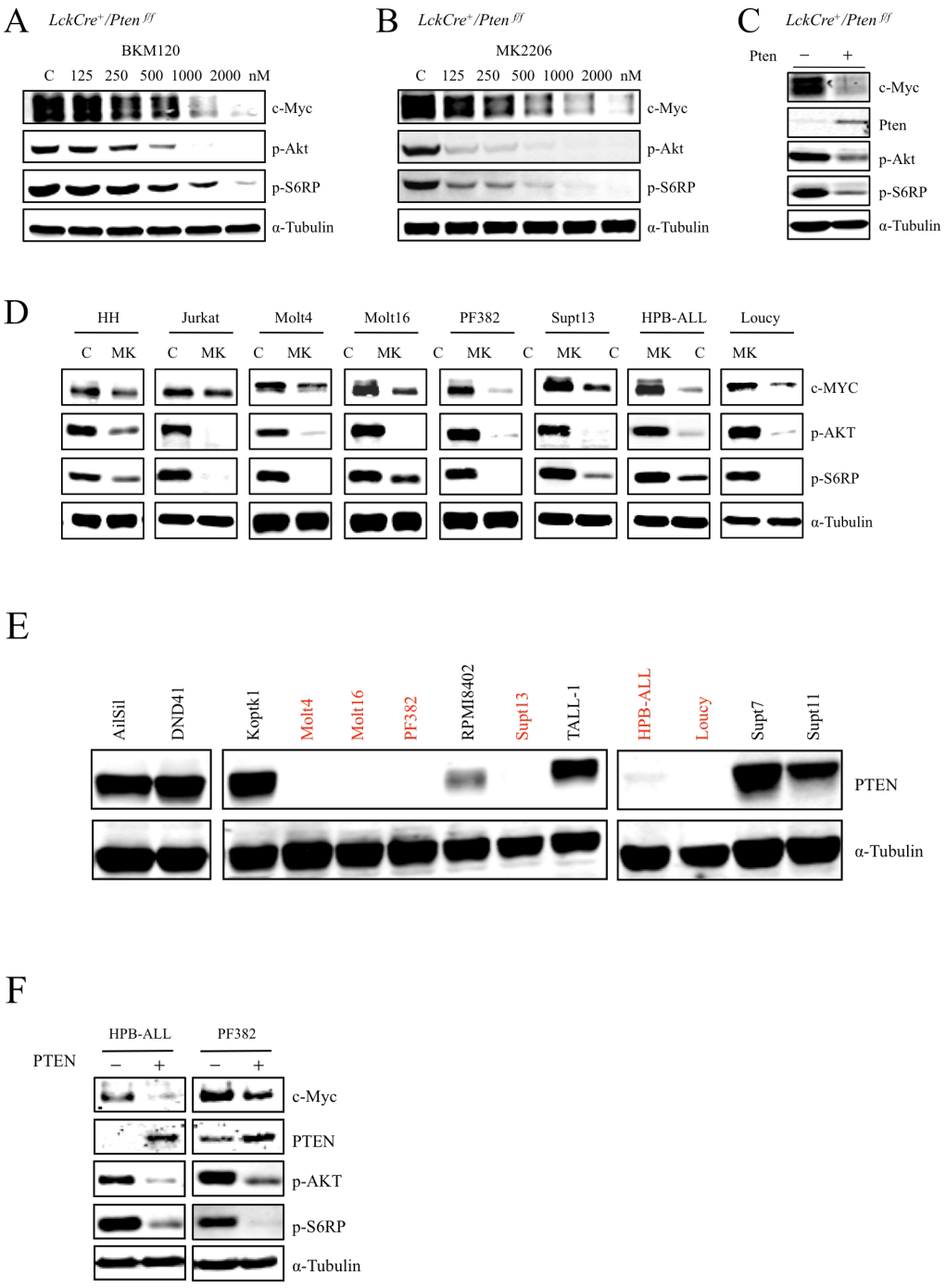
(D) The abundances of both c-MYC protein and PI3K signaling in human PTEN-deficient T-ALL cells are reduced upon one hour treatment with MK2206.

(E) The abundances of both c-MYC protein and PI3K signaling in human PTENdeficient T-ALL cells are reduced upon one hour treatment with MK2206.

(F) Ectopic expression of PTEN suppressed c-MYC protein level and PI3K signaling in PTEN deficient T-ALL cell lines HPB-ALL and PF382.

Contributions: I performed all the experiments in this figure.

Figure 3.3 (Continued)



Contributions: I performed all the experiments in this figure.

We next sought to understand the nature of the regulation of c-Myc by the PI3K-Akt pathway activation. We first determined that gene expression levels of *Myc* are increased in our *LckCre*$^{+}$*/Pten*$^{f/f}$ primary tumor cells (Fig 3.4A), consistent with previously described *Pten*-null T-ALL models (Guo et al., 2008; Liu et al., 2010; Zhang et al., 2011) Interestingly, while PI3K/Akt inhibition caused a dramatic decrease of c-Myc protein abundance (Fig 3.3), the level of *Myc* mRNA in these cancer cells was not altered upon treatment with the AKT inhibitor, MK2206 (Fig 3.4B), suggesting that the gene expression of *Myc* is not affected by PI3K/Akt activity.

It has been reported that, GSK3β, a downstream effector of AKT, can regulate c-MYC protein stability through phosphorylation of c-MYC at the threonine-58 residue (T58) and subsequent proteasome-mediated degradation (Sears et al., 2000). To test whether this mechanism is also functioning in our T-ALL model, we treated the primary T-ALL cells with the translation elongation inhibitor cycloheximide (CHX) either alone or in combination with MK2206. Application of CHX suppresses biosynthesis and as a result, c-Myc protein levels quickly decreased due to the protein's short half-life. Addition of MK2206, however, failed to either expedite or postpone the degradation of c-Myc (Fig 3.4C). These results indicate that PI3K/Akt signaling has little effect on the stability of c-Myc protein in these T-ALL cells.

We next tested whether the PI3K pathway regulates *de novo* synthesis of the c-Myc protein. *LckCre*$^{+}$*/Pten*$^{f/f}$ primary tumor cells were pre-treated with CHX to deplete c-Myc protein, and CHX was subsequently washed away, quickly followed by the addition of

MG132, a proteasome inhibitor, to preserve newly synthesized c-Myc. As expected, newly synthesized c-Myc was stabilized and accumulated in MG132 treated cells, but not in cells treated with MG132 plus CHX (Fig 3.4D). Strikingly, little c-Myc protein was detected in cells treated with MG132 plus MK2206 (Fig 3.4D), suggesting that Akt inhibition profoundly blocked *de novo* synthesis of c-Myc.

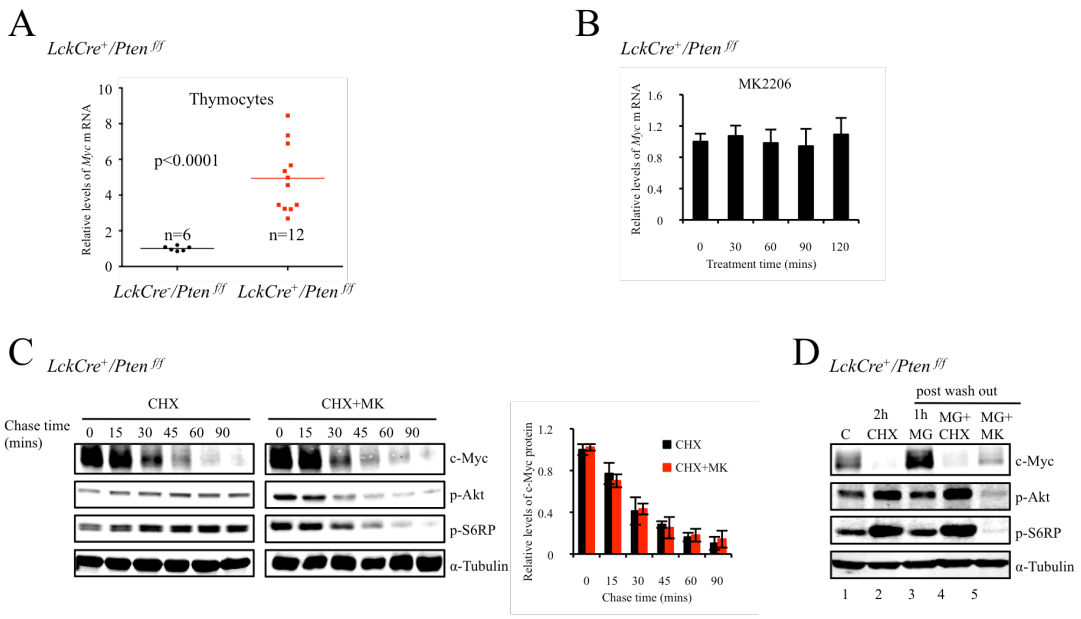


Figure 3.4 The PI3K-mTORC1-S6K1 Signaling Regulates c-Myc *de novo* Synthesis in Pten-Deficient T-ALL

(A) The expression levels of c-Myc mRNA in the thymocytes of *LckCre*$^{+}$*/Pten*$^{f/f}$ moribund mice are significantly higher than that of age matched *LckCre*$^{-}$*/Pten*$^{f/f}$ healthy mice.

Figure 3.4 (Continued)

(B) The expression levels of c-Myc mRNA in primary T-ALL cells derived from *LckCre*$^{+}$*/Pten*$^{f/f}$ mice remain unchanged upon 1μM MK2206 treatment at the indicated time points. c-Myc mRNA levels were measured by quantitative PCR. GAPDH was used as control.

(C) The c-Myc protein stability in primary T-ALL cells derived from *LckCre*$^{+}$*/Pten*$^{f/f}$ mice remained unchanged upon MK2206 treatment for the indicated time points. Cells treated with 25μg/ml cycloheximide (CHX) with or without 1μM MK2206 are indicated.

(D) AKT inhibition blocked the *de novo* synthesis of c-Myc protein. *LckCre*$^{+}$*/Pten*$^{f/f}$ primary tumor cells (lane 1) were pre-treated with 25μg/ml CHX for two hours to deplete c-Myc protein (lane 2) and cells were washed three times with PBS to allow new biosynthesis. MG132 was then added at 10μM either alone (lane 3) or with 25μg/ml CHX (lane 4) or with 1μM MK2206 (lane 5) for one hour.

We next set out to examine the mechanism by which the PI3K/Akt pathway regulates c-Myc translation in *LckCre*$^{+}$*/Pten*$^{f/f}$ primary tumor cells. We first tested the effect of the inhibition of mTORC1, an essential downstream effector of PI3K/Akt known to be critical for translational control, on the c-Myc protein abundance in these cells. We treated the *LckCre*$^{+}$*/Pten*$^{f/f}$ primary tumor cells with an mTORC1 inhibitor RAD001 or the PI3K/mTORC1 dual inhibitor BEZ235 (Maira et al., 2008; Schuler et al., 1997) and

found that both inhibitors indeed dramatically suppressed c-Myc protein levels and the downstream signaling of their targets (Fig 3.5A-3.5B). The growth of these primary tumor cells was also markedly reduced in a dose-dependent manner (Fig 3.5A-3.5B). Similar to MK2206 described above, neither BEZ235 nor RAD001 affected *Myc* transcription or degradation in primary cells (Fig 3.5C-3.5D). To determine whether the phenotype we observed *in vitro* can be replicated *in vivo*, we measured the ability of the PI3K/mTORC1 dual inhibitor BEZ235 (Maira et al., 2008) to suppress tumor growth and c-Myc protein levels *in vivo* (Fig 3.5E-3.5G).

Figure 3.5 The mTORC1-S6K Signaling Regulates c-Myc *de novo* Synthesis in Pten-Deficient T-ALL

(A-B) PI3K or mTORC1 inhibition by RAD001 or BEZ235 treatment as indicated reduced both the growth and c-Myc protein abundance in *LckCre+/Ptenf/f* primary tumor cells. For growth inhibition, indicated drugs were applied at indicated concentrations for indicated hours before measurement; for Western blotting, cells were treated with indicated drugs for two hours. DMSO was used as negative control.

(C) The expression levels of c-Myc mRNA in primary T-ALL cells derived from *LckCre+/Ptenf/f* mice remain unchanged upon 100nM BEZ235 or 100nM RAD001 treatment at the indicated time points. c-Myc mRNA levels were measured by quantitative PCR. GAPDH was used as control.

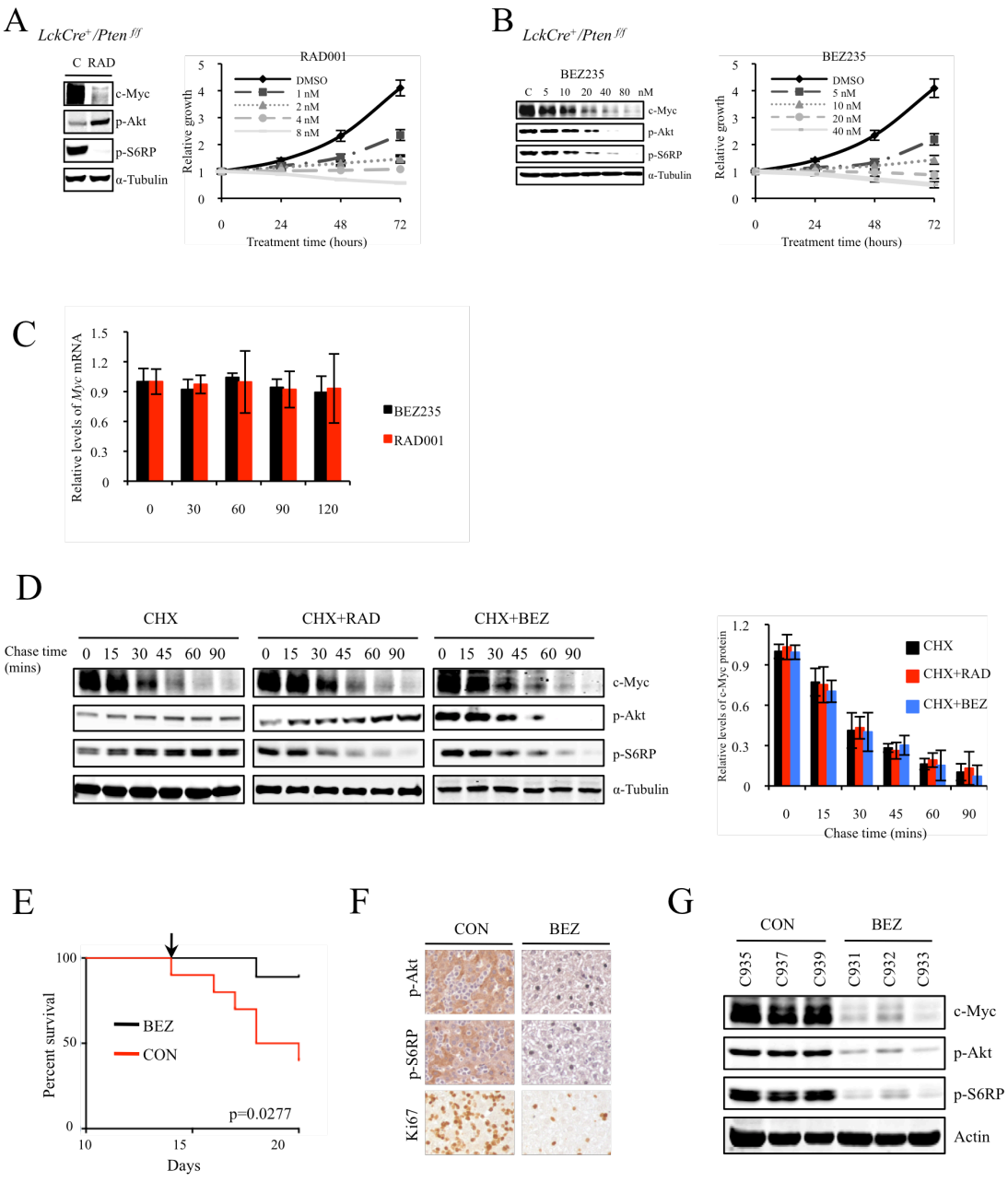
(D) The c-Myc protein stability in primary T-ALL cells derived from *LckCre+/Ptenf/f* mice remained unchanged upon RAD001 or BEZ235 treatment for the indicated time points. Cells treated with 25μg/ml cycloheximide (CHX) alone or with 100nM RAD001, or 100nM BEZ235 are indicated.

(E) Recipient mice transplanted with two million *LckCre+/Ptenf/f* moribund mice thymocytes showed prolonged survival curve with daily BEZ235 treatment (35mg/kg) compared to control treatment. Arrow indicates the starting date of treatment (day 14) post transplantation.

Figure 3.5 (Continued)

(F-G) Recipient mice transplanted with two million *LckCre+/Ptenf/f* moribund mice thymocytes showed suppressed PI3K signaling as measured by p-Akt (S473), p-S6RP (S235/236) IHC staining **(F)**, deceased proliferation rate as measured by Ki67 IHC staining **(F)** and decreased c-Myc protein levels as detected by Western blotting **(G)** after three days of BEZ235 daily treatment (35mg/kg), starting at 14 days post transplantation. PEG300 was used as control treatment for in vivo inhibitor administration.

Figure 3.5 (Continued)



Contributions: Thanh Von, Dr. Lukas Baitsch and Dr. Haluk Yuzugullu assisted with mice handling and FACS analysis for in this figure. I performed the rest of the experiments for this figure.

While mTORC1 has been previously implicated in regulating c-MYC, the mechanism remains unclear. The traditional model is that mTORC1 stimulates translation by phosphorylating the eIF4E-binding protein (4EBP1), which blocks the inhibitory interaction of 4EBP1 with eIF4E, rending eIF4E available for binding to eIF4G and thus recognition of the 5'-cap. Given the well-established significance of assembling the eIF4E/eIF4G complex in translation initiation, we first tested whether a small molecule inhibitor termed 4EGI-1 that blocks the interaction of eIF4E with eIF4G could reduce c-Myc protein abundance as we have recently shown that 4EGI-1 significantly reduced the c-MYC protein translation in human melanoma and breast cancer cells. To our surprise, 4EGI-1 treatment greatly increased c-Myc protein abundance in these T-ALL cells (Fig. 3.6A). Interestingly, 4EGI-1 also increased pS6RP levels (Fig. 3.6A). To investigate whether S6K1 plays a role in regulating c-Myc protein in our *Pten*-null T-ALL model, we utilized a small molecule inhibitor of S6K1, PF470861. Notably S6K1 inhibition effectively decreased c-Myc protein levels and inhibited tumor cell proliferation in a dose-dependent manner (Fig 3.6B). Similar to the inhibition of its upstream activators, S6K1 inhibition did not affect gene transcription or protein stability of c-Myc (Fig 3.6C-3.6D). Consistent with the treatment of MK2206, application of either RAD001 or BEZ235, or PF470861 markedly prevented c-Myc protein *de novo* synthesis in *LckCre*$^{+}$*/Pten*$^{f/f}$ primary tumor cells (Fig 3.6E). Together, our results indicate the mTORC1-S6K1 axis downstream of PI3K/Akt plays a critical role in the regulation of c-Myc *de novo* synthesis.

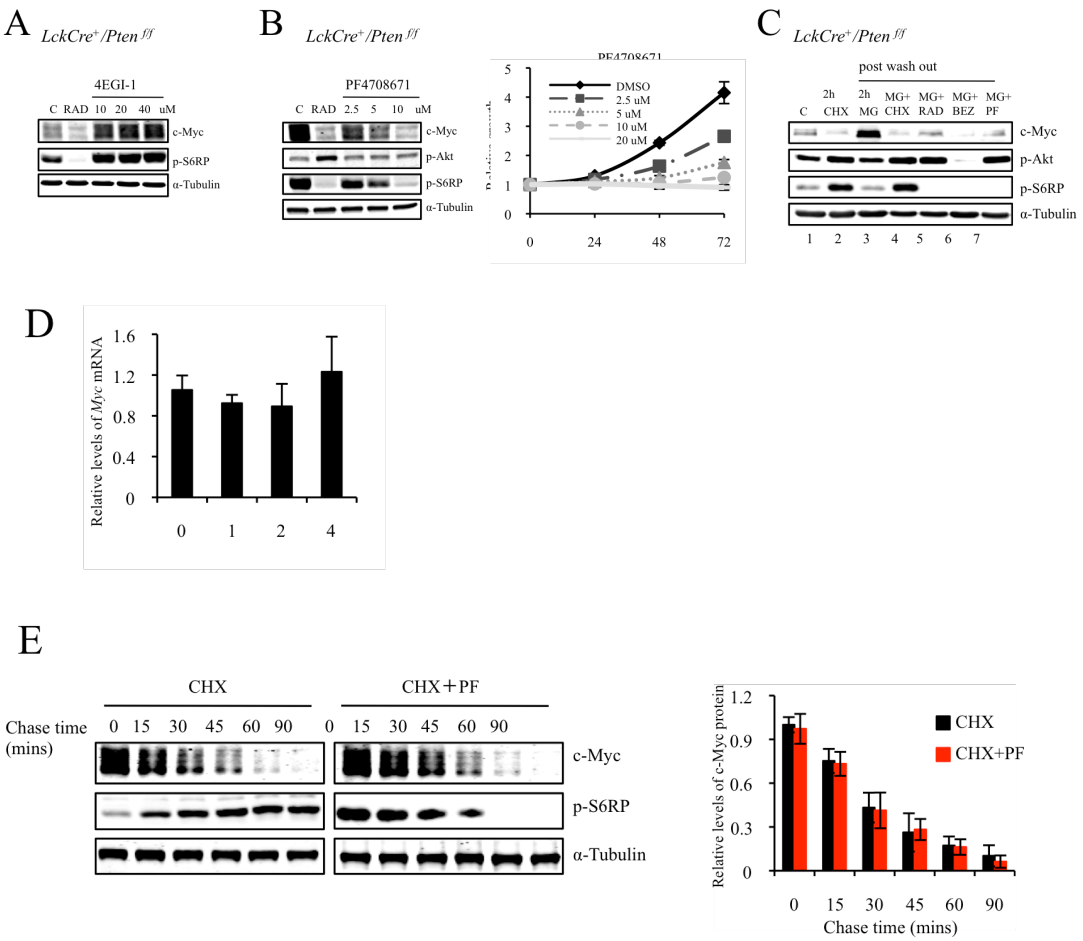


Figure 3.6 The S6K1 Downstream of PI3K/mTORC1 Regulates c-Myc *de novo* Synthesis

(A) Inhibition of eIF4E and eIF4G interaction by 4EGI-1 increased pS6RP and c-Myc protein level. Cells were treated with 4EGI-1 at indicated concentrations for four hours. DMSO was used as negative control.

Figure 3.6 (Continued)

(B) S6K1 inhibition by PF4708671 treatment as indicated reduced both the growth and c-Myc protein abundance in *LckCre+/Ptenf/f* primary tumor cells. For growth inhibition, PF4708671 was applied at indicated concentrations for indicated hours before measurement; for Western blotting, cells were treated with indicated drugs for two hours. DMSO was used as negative control.

(C) PI3K, mTORC1 or S6K1 inhibition by RAD001, BEZ230 or PF4708671 treatment as indicated blocked the *de novo* synthesis of c-Myc protein. *LckCre+/Ptenf/f* primary tumor cells (lane 1) were pre-treated with 25μg/ml CHX for two hours to deplete c-Myc protein (lane 2) and cells were washed three times with PBS to allow new biosynthesis. MG132 was then added at 10μM either alone (lane 3), or with 25μg/ml CHX (lane 4), or with 100 nM RAD001 (lane 5), or with 100nM BEZ235 (lane 6), or with 10μM PF4708671 (lane 7) for two hours.

(D) The expression levels of c-Myc mRNA in primary T-ALL cells derived from *LckCre+/Ptenf/f* mice remain unchanged upon 10μM PF4708671 treatment at the indicated time points. c-Myc mRNA levels were measured by quantitative PCR. GAPDH was used as control.

Figure 3.6 (Continued)

(E) The c-Myc protein stability in primary T-ALL cells derived from *LckCre+/Ptenf/f* mice remained unchanged upon treatment for the indicated time points. Cells treated with 25μg/ml cycloheximide (CHX) with or without 10μM PF4708671 are indicated.

Contributions: I performed all the experiments for this figure.

We have shown that that c-MYC protein levels were inhibited by application of the AKT inhibitor MK2206 in a panel of human PTEN-deficient T-ALL cell lines (Fig 3.3). To determine whether mTORC1 can also regulate c-MYC in human PTEN-deficient T-ALLs, we treated the previously described eight cell lines (Fig 3.3) with RAD001. Similar to the inhibition of AKT, mTORC1 inhibition in all these cell lines decreased c-MYC protein abundance (Fig 3.7A). We then focused our subsequent studies in two PTEN-deficient, c-MYC high T-ALL cell lines, HPB-ALL and PF382. Similar to the inhibition of mTORC1, inhibition of S6K1 also greatly suppressed c-MYC protein levels in HPB-ALL and PF382 (Fig 3.7B-3.7C). Consistent with our findings in the primary cells, the application of MK2206, or RAD001, or PF470861 had little effect on neither gene expression of *MYC* (Fig 3.7D-3.7E) or c-MYC protein stability (Fig 3.7F-3.7G) in both cell lines. Impressively, all these inhibitors targeting the AKT-mTORC1-S6K1 axis showed strong inhibition of the *de novo* sysnthesis of c-MYC protein (Fig 3.7H-3.7I), Notably, inhibition of the eIF4E/eIF4G interaction by 4EGI-1 in these human PTEN-deficient T-ALL cells also led to the same perplexing results seen in mouse primary Pten-

null T-ALL cells: increased levels of c-MYC protein and pS6RP (Fig. 3J), suggesting that the translation of c-MYC in these leukemic cells is uniquely regulated by the PI3K/mTOR/S6K pathway activation.

Figure 3.7 The mTORC1-S6K1 Axis Regulates the *de novo* Synthesis of c-MYC Protein in Human PTEN-Deficient T-ALL Cell Lines

(A) The abundances of both c-MYC protein and PI3K signaling in human PTENdeficient T-ALL cells are reduced upon one hour treatment with MK2206 or RAD001.

(B-C) The abundances of c-MYC protein were reduced upon RAD001 or PF4708671 treatment in HPB-ALL and PF382 cells. DMSO treatment was used as negative control.

(D-E) Inhibition of AKT/mTORC1/S6K1 did not change the level of *MYC* transcription. HPB-ALL cells **(D)** or PF382 cells **(E)** were treated with MK2206, RAD001 or PF4708671 for one to four hours as indicated. c-MYC mRNA levels were measured by quantitative PCR. GAPDH was used as control.

(F-G) Inhibition of AKT/mTOR/S6K1 did not affect c-MYC protein stability. HPB-ALL cells **(F)** or PF382 cells **(G)** were treated with 25μg/ml cycloheximide (CHX) with or without MK2206, RAD001 or PF4708671 for the indicated time points. Cell lysates were subjected to Western blotting, followed by quantification.

Figure 3.7 (Continued)

(H-I) The *de novo* synthesis of c-MYC protein was inhibited by AKT/mTORC1/S6K1 inhibition. HPB-ALL **(H)** or PF382 **(I)** cells (lanes 1) were pre-treated with 25μg/ml CHX for two hours to deplete c-Myc protein (lanes 2) and cells were washed three times with PBS to allow new biosynthesis. MG132 was then added either alone (lanes 3) or with CHX (lanes 4) or with MK2206 (lanes 5), or with RAD001 (lanes 6), or with PF4708671 (lanes 7) for two hours.

(J) Inhibition of eIF4E and eIF4G interaction by 4EGI-1 increased pS6RP and c-Myc protein level. HPB-ALL cells were treated with 4EGI-1 at indicated concentrations for four hours. DMSO was used as negative control.

Figure 3.7 (Continued)

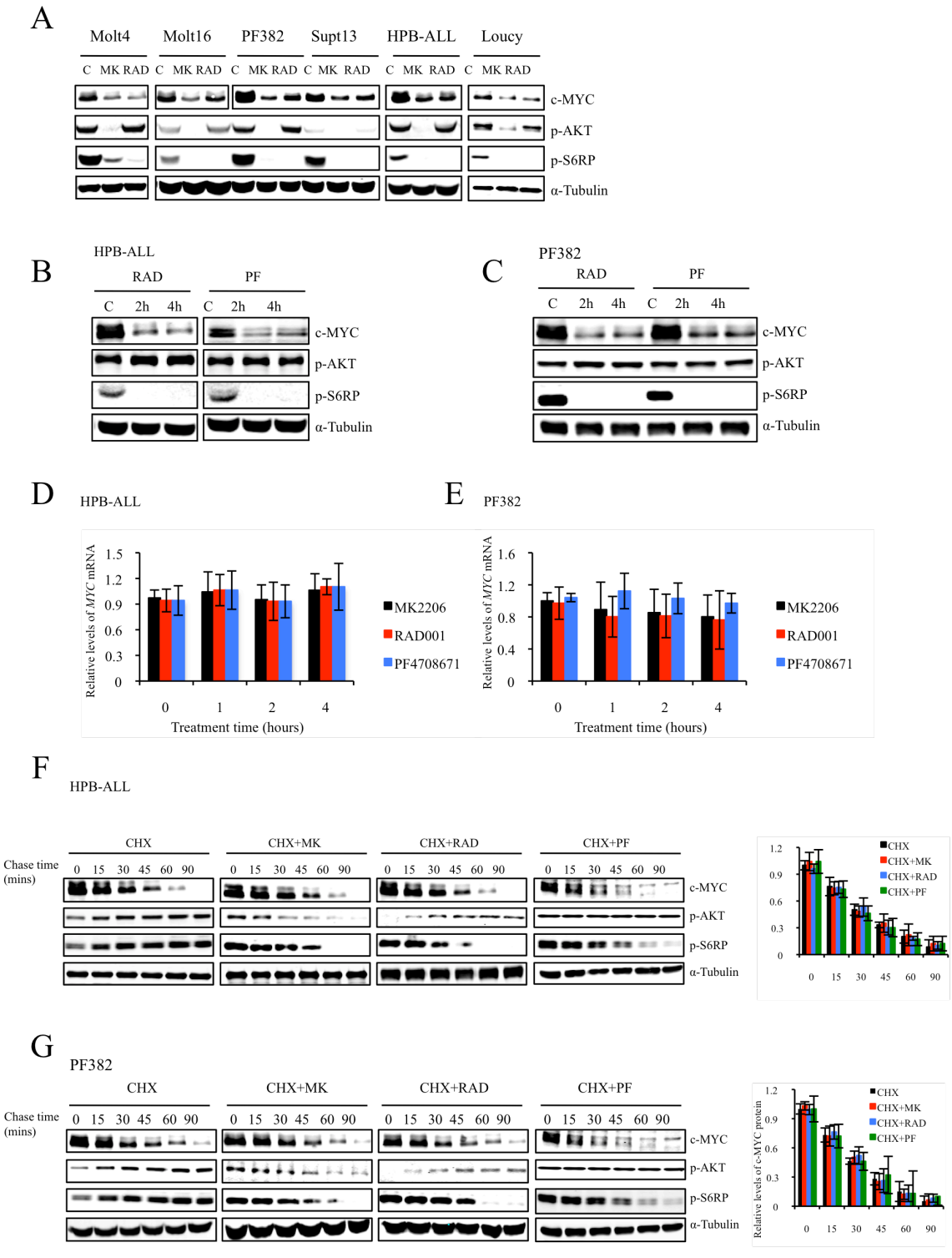
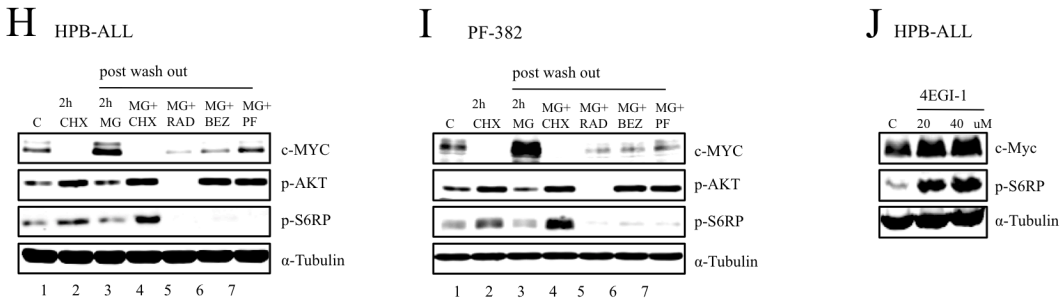


Figure 3.7 (Continued)



We next investigated how mTORC1/S6K1 regulates c-MYC protein synthesis. The mRNA of *MYC* contains a highly structured 5' untranslated region (5'UTR) (Stoneley et al., 1998) that has been implicated in regulating its translation (Galmozzi et al., 2004). To test whether the *MYC* 5'UTR is critical for the translational control of c-MYC by the PI3K/mTORC1/S6K1 pathway, we used a luciferase construct in which the SV40 promoter drives firefly luciferase expression either with or without a *MYC* 5'UTR (Fig 3.8A). A renilla luciferase construct was used as internal control. We tested the response of luciferase report activity to inhibitors targeting the PI3K pathway. Notably, treatment with either BEZ235 or PF470861 significantly reduced the luciferase activity expressed from the construct containing the *MYC* 5'UTR, but not the one lacking a *MYC* 5'UTR (Fig 3.8A). These data suggest that the PI3K/mTORC1/S6K1 pathway regulates *MYC* translation via its structured 5'UTR.

To further validate whether *MYC* 5'UTR is important for its translation regulated by the PI3K/mTORC1/S6K1 signaling axis in PTEN-deficient T-ALL, we utilized a retroviral expression vector to introduce a stabilized mutant allele of *MYC*, *MYC* T58A, with or

without the *MYC* 5'UTR, into HPB-ALL cells, resulting in a pair of isogenic lines: HPB-ALL *MYC* T58A -5'UTR and HPB-ALL *MYC* T58A +5'UTR. Strikingly, while the abundance of c-MYC protein in HPB-ALL *MYC* T58A -5'UTR cells was little changed in response to either BEZ235 or PF4708671, it was markedly reduced in cells expressing HPB-ALL *MYC* T58A +5'UTR in response to inhibitors treatment despite the presence of the stabilizing mutant c-MYC T58A (Fig 3.8B-3.8C). Notably, the abundance of c-MYC protein in the parental cells expressing vector control also dramatically reduced by the inhibitor treatments (Fig 3.8B-3.8C), suggesting that endogenous *MYC* mRNAs in these T-ALL cells carrying 5'UTR are subjected to the regulation by the PI3K/mTORC1/S6K activity. We have further confirmed that the 5'UTR is present in the *Myc* mRNA transcripts in the *Pten*-null T-ALL derived from $LckCre^{+}/Pten^{f/f}$ mice as well as in a panel of human PTEN-deficient c-MYC high T-ALL lines that we have tested.

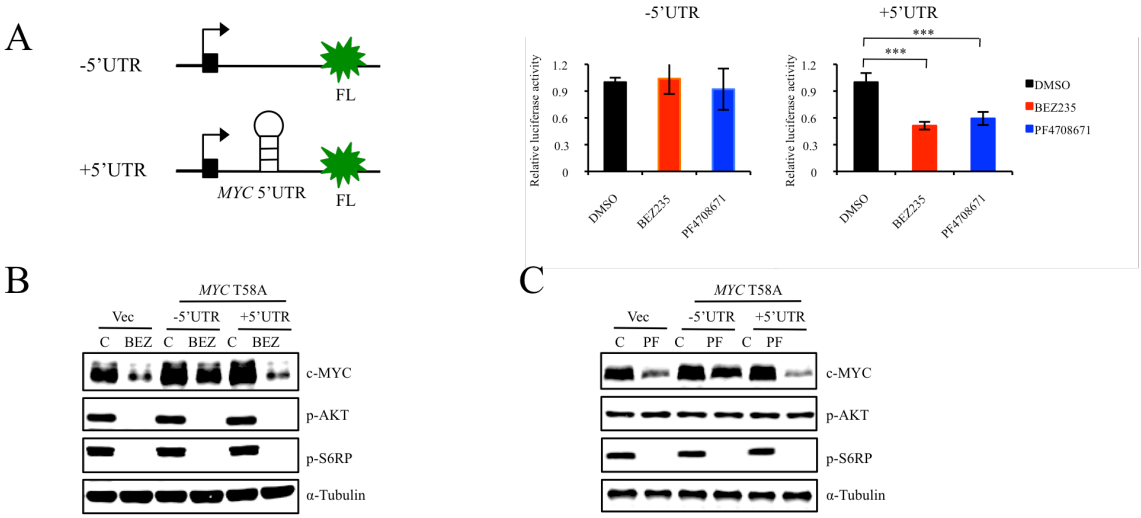


Figure 3.8 The PI3K/mTOR/S6K1 Signaling Regulates c-MYC 5'UTR

(A) The activity of firefly luciferase reporter in the construct carrying a *MYC* 5'UTR is sensitive to the inhibition of PI3K/mTORC1 or S6K1. Normalized units of luciferase activities in the cell lysates from co-transfection of the pGL3 with or without the 5'UTR of human *MYC* and the pRL renilla luciferase reporter as internal control are shown. Luciferase activities were measured in cells treated with BEZ235 or PF4708671 for four hours two days post transfection. ***P< 0.0005 (Student's t test).

(B-C) The differential responses of c-MYC protein abundance in HPB-ALL cells expressing vector control, *MYC* T58A -5'UTR or *MYC* T58A +5'UTR upon BEZ235 or PF4708671 treatment as indicated.

Since the ectopically expressed *MYC* T58A constructs in this experiment had no tag attached, their protein bands were superimposed with endogenous c-MYC in the western blotting analysis (Fig 3.5B-3.5C). We then expressed a Flag-tagged *MYC* T58A -5'UTR in HPB-ALL and a number of additional T-ALL cell lines to display a Flag-tagged c-MYC T58A that is distinct from the endogenous c-MYC (Fig 3.9A-3.9E). Our results clearly show that, while the abundance of the Flag-tagged c-MYC T58A protein in cells remained unchanged in response to PI3K, AKT or mTORC1 inhibitors, the amount of endogenous c-MYC reduced dramatically upon treatment with these drugs (Fig 3.9A-3.9E). Consistent with this finding, HPB-ALL *MYC* T58A -5'UTR cells have significantly reduced sensitivity to growth inhibition upon treatment with PI3K/mTORC1 or S6K1 inhibitors as compared to parental cells or the isogenic HPB-ALL *MYC* T58A +5'UTR cells (Fig 3.9F-3.9J). The fact that growth inhibition of PI3K/mTORC1 and S6K1 inhibitors is not fully rescued by expressing the exogenous c-MYC suggest that in addition to c-MYC, other downstream targets were also regulated by PI3K/mTORC1 and S6K1 signaling. Together, these data demonstrate that the PI3K/mTORC1/S6K1 signaling axis governs the translation of *MYC* mRNAs bearing 5'UTR.

Figure 3.9 The PI3K/mTOR/S6K1 Signaling Regulates the 5'UTR-Dependent Translation of c-Myc.

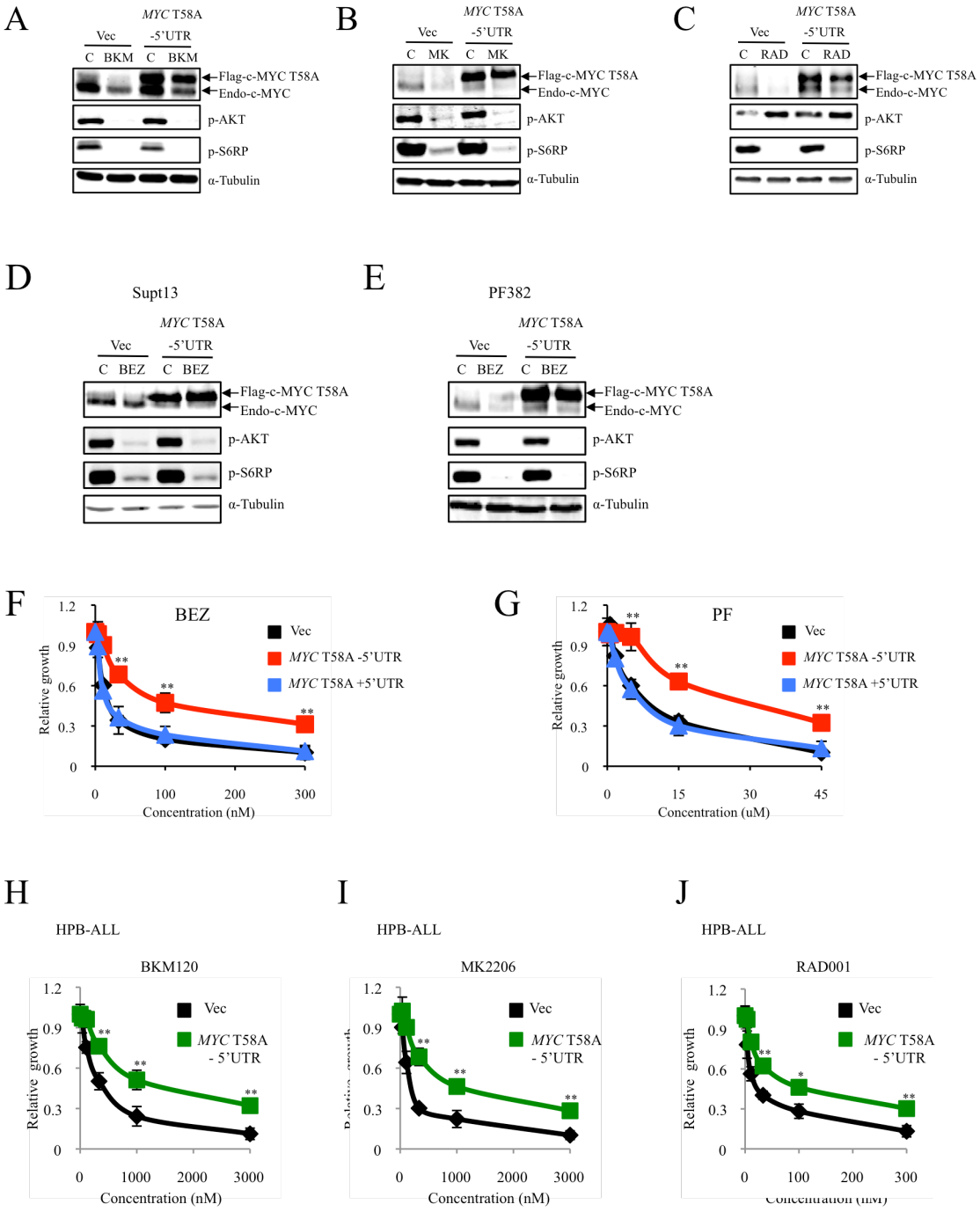
(A-C) The response of Flag-tagged c-MYC T58A protein or endogenous c-MYC protein abundance in HPB-ALL cells upon BKM120, MK2206 or RAD001 treatment as indicated.

(D-E) The response of Flag-tagged c-MYC T58A protein or endogenous c-MYC protein abundance in PTEN-deficient T-ALL cells Supt13 **(D)**, and PF382 **(E)** upon BEZ235 treatment as indicated.

(F-G) The differential responses of the growth rate of HPB-ALL cells expressing vector, *MYC* T58A -5'UTR, or *MYC* T58A +5'UTR as indicated upon BEZ235 or PF4708671 treatment. $^{**}P < 0.005$ (Student's t test).

(H-J) The differential responses of the growth rate of HPB-ALL cells expressing vector, or *MYC* T58A -5'UTR as indicated upon BKM120 **(H)**, MK2206 **(I)**, or RAD001 **(J)** treatment. $^{*}P<0.05$, $^{**}P< 0.005$ (Student's t test).

Figure 3.9 (Continued)



Previous studies have shown that S6K1 can phosphorylate the translation initiation factor eIF4B (Raught et al., 2004), which has been implicated in facilitating the helicase activity of the dead-box RNA helicase eIF4A (Andreou and Klostermeier, 2013; Rogers et al., 1999). eIF4A has been shown to be important in the unwinding of structured 5'UTRs for translation initiation (Lawson et al., 1986; Parsyan et al., 2011). To test whether eIF4B and elF4A are involved in the translational regulation of *MYC* in our leukemic cells, we introduced multiple shRNAs against eIF4B or eIF4A into HPB-ALL cells. Notably, the knock-down efficiency of either eIF4B or eIF4A closely correlated with both the reduction of c-MYC protein levels in these cells, as well as the reduction of tumor cell proliferation (Fig 3.10A-3.10D), suggesting that both eIF4B and elF4A are important in regulating the translation of c-MYC. Taken together, our results demonstrate that in PTEN-deficient and c-MYC overespressing T-ALL cells, the hyper-activated PI3K/mTORC1/S6K1 signaling axis is critical for the translation of elevated number of *MYC* transcripts bearing 5'UTR via an eIF4B/eIF4A mediated translational mechanism.

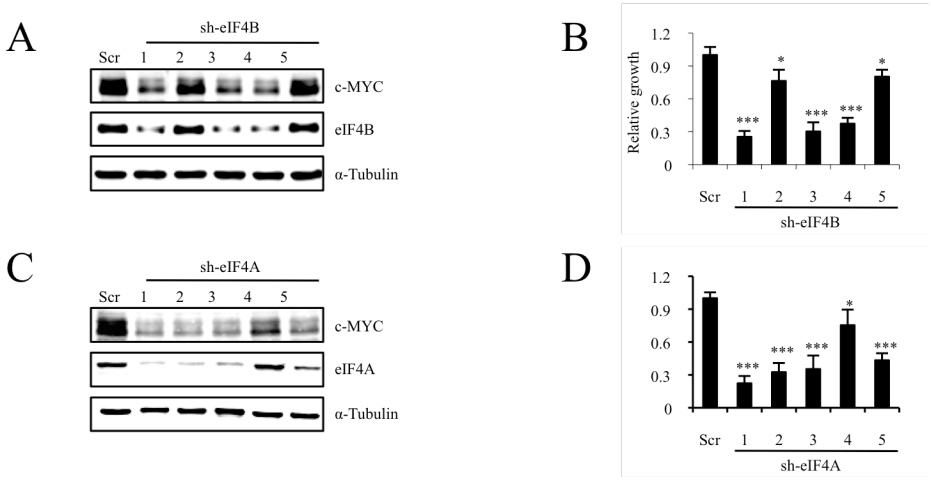


Figure 3.10 Translation Initiation Factor eIF4A/eIF4B Regulates c-MYC and Tumor Cell Proliferation

(A-B) The change of the abundance of c-MYC protein and growth rate of HPB-ALL cells in response to shRNA-mediated knockdown of eIF4B. Cells expressing scramble control (Scr), or five independent shRNA constructs against eIF4B as indicated. **P*< 0.05, ****P*<0.0005 (n=3, Student's t test).

(C-D) The change of the abundance of c-MYC protein and growth rate of HPB-ALL cells in response to shRNA-mediated knockdown of eIF4A. Cells expressing scramble control (Scr), or five independent shRNA constructs against eIF4A, as indicated. **P*< 0.05, ****P*<0.0005 (n=3, Student's t test).

Contributions: I performed all the experiments for the figure.

We next examined whether the helicase activity of eIF4A is important for the translation control of *MYC* using hippuristanol, a selective enzymatic inhibitor of eIF4A (Bordeleau et al., 2006). We first tested primary *Pten*-null T-ALL cells isolated from our *LckCre*$^{+}$*/Pten*$^{f/f}$ mice and found that hippuristanol effectively reduced the c-Myc protein levels and the growth of these primary tumor cells in a dose dependent manner (Fig 3.11A-3.11B). We further confirmed that hippuristanol did not affect *Myc* transcription or degradation (Fig 3.11C-3.11D), but instead strongly blocked *Myc* translation (Fig 3.11E).

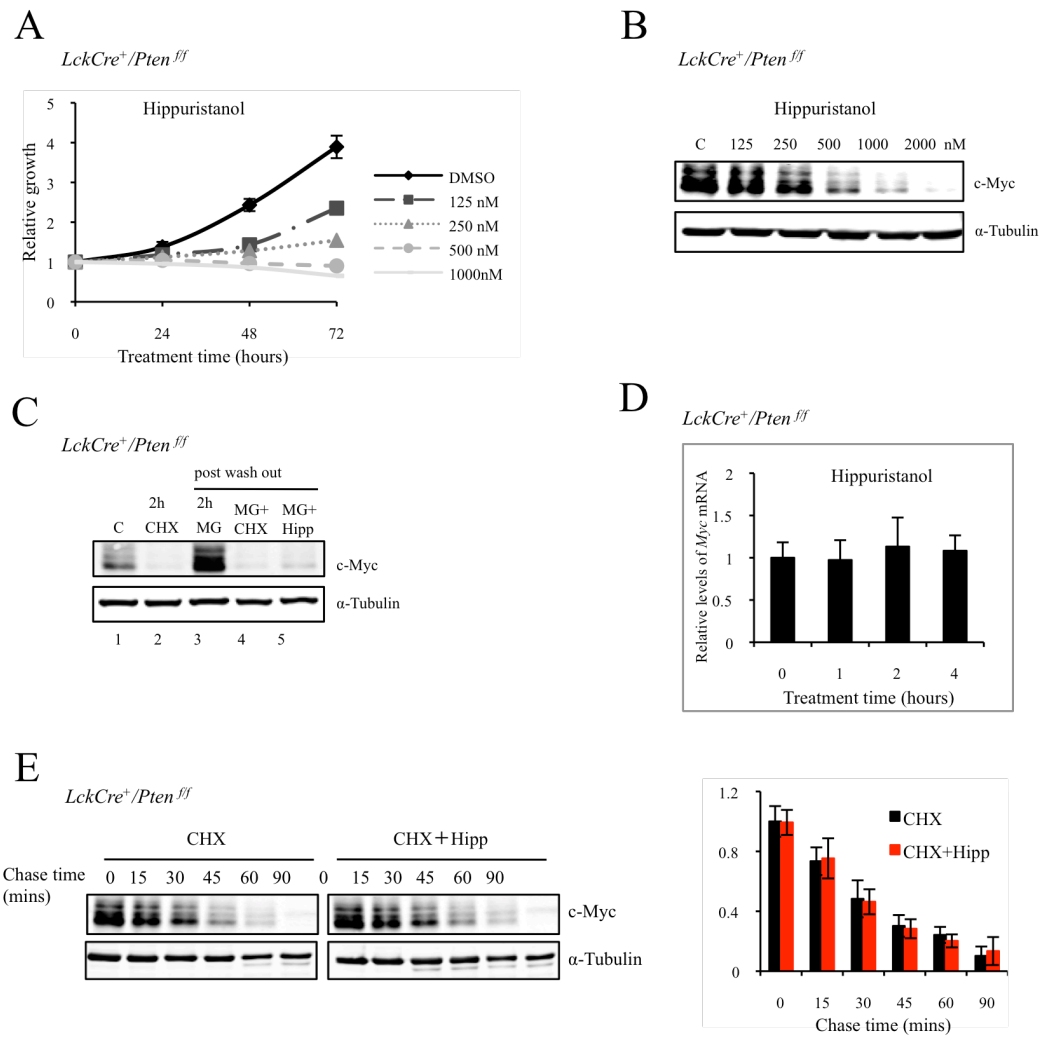


Figure 3.11 Hippuristanol, a Small Molecule Inhibitor of eIF4A, Blocks the Translation of c-MYC in T-ALL

(A) The response of the growth rate of primary *LckCre+/Ptenf/f* T-ALL cells upon hippuristanol treatment. Hippuristanol was applied to cells at the indicated concentrations for indicated hours before measurement.

Figure 3.11 (Continued)

(B) The response of the abundance of c-Myc in primary *LckCre+/Ptenf/f* T-ALL cells upon hippuristanol treatment. Hippuristanol was applied to cells at the indicated concentrations for two hours. DMSO treatment was used as negative control.

(C) The *de novo* synthesis of c-Myc protein in primary *LckCre+/Ptenf/f* T-ALL cells is inhibited by hippuristanol treatment. Cells were pre-treated with 25μg/ml CHX for two hours to deplete c-Myc protein (lanes 1-2), and subsequently washed three times with PBS to allow new biosynthesis. MG132 was then added at 10μM either alone (lanes 3) or with 25μg/ml CHX (lanes 4) or with 1μM hippuristanol (lanes 5) for two hours.

(D) Inhibition of eIF4A helicase activity did not change the level of *MYC* transcription. Primary *LckCre+/Ptenf/f* T-ALL cells were treated with hippuristanol for one to four hours as indicated. c-MYC mRNA levels were measured by quantitative PCR. GAPDH was used as control.

(E) Inhibition of eIF4A helicase activity did not affect c-MYC protein stability. Primary *LckCre+/Ptenf/f* T-ALL cells were treated with 25μg/ml cycloheximide (CHX) with or without hippuristanol for the indicated time points. Cell lysates were subjected to Western blotting, followed by quantification.

Again we extended the experiment from our murine primary T-ALL model to human cancer cell lines. Consistent with the findings in primary *LckCre*$^{+}$*/Pten*$^{f/f}$ T-ALL, hippuristanol greatly reduced c-MYC protein levels in c-MYC dependent human T-ALL cell lines in a dose-dependent manner (Fig 3.12A-3.12B). We also confirmed that hippuristanol prevented c-MYC translation without affecting its transcription or degradation (Fig 3.12C-3.12E).

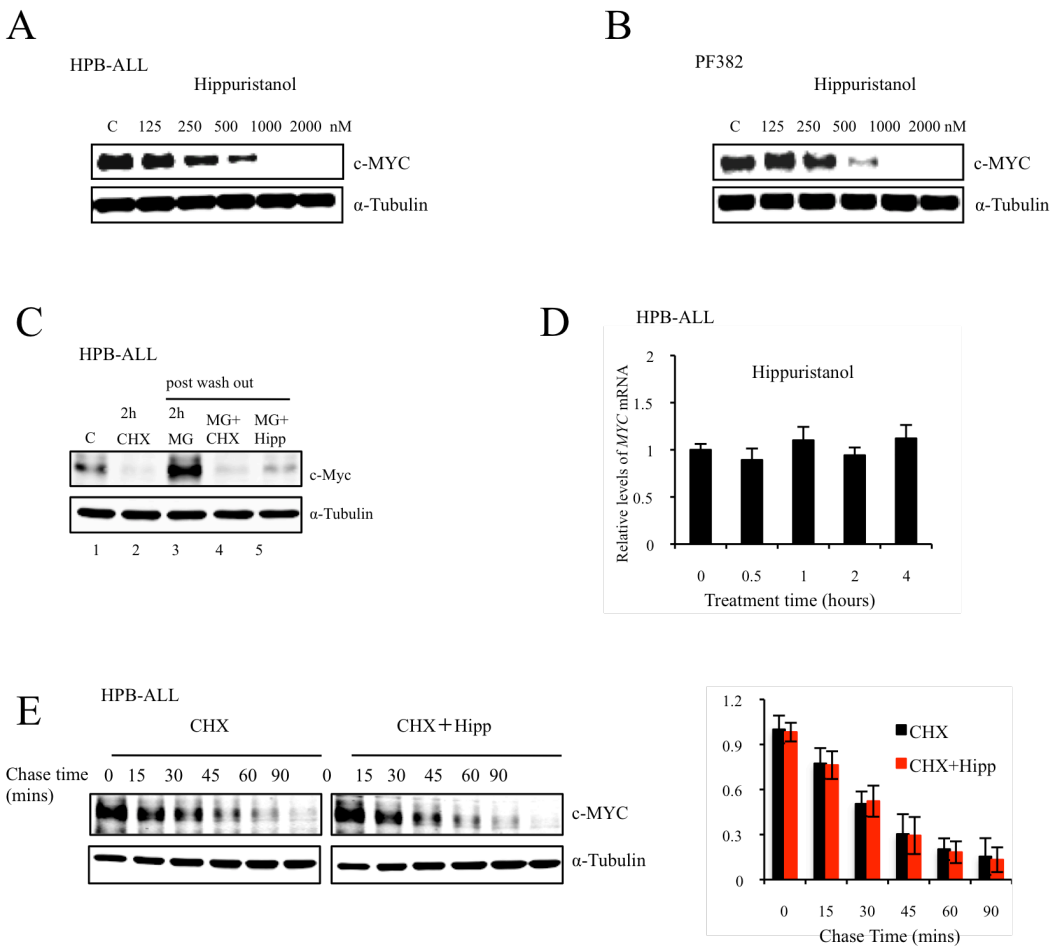


Figure 3.12 Hippuristanol Blocks the Translation of c-MYC in human PTENdeficient T-ALL cell lines.

(A-B) The response of the abundance of c-Myc in HPB-ALL **(A)** and PF382 **(B)** cells upon hippuristanol treatment. Hippuristanol was applied to cells at the indicated concentrations for two hours. DMSO treatment was used as negative control.

Figure 3.12 (Continued)

(C) The *de novo* synthesis of c-Myc protein in HPB-ALL cells is inhibited by hippuristanol treatment. Cells were pre-treated with 25μg/ml CHX for two hours to deplete c-Myc protein (lanes 1-2), and subsequently washed three times with PBS to allow new biosynthesis. MG132 was then added at 10μM either alone (lanes 3) or with 25μg/ml CHX (lanes 4) or with 1μM hippuristanol (lanes 5) for two hours.

(D) Inhibition of eIF4A helicase activity did not change the level of *MYC* transcription. HPB-ALL cells were treated with hippuristanol for one to four hours as indicated. c-MYC mRNA levels were measured by quantitative PCR. GAPDH was used as control.

(E) Inhibition of eIF4A helicase activity did not affect c-MYC protein stability. HPBALL cells were treated with 25μg/ml cycloheximide (CHX) with or without hippuristanol for the indicated time points. Cell lysates were subjected to Western blotting, followed by quantification.

To determine whether hippuristanol selectively targets the *MYC* 5'UTR, we treated the isogenic cell lines HPB-ALL *MYC* T58A -5'UTR and HPB-ALL *MYC* T58A +5'UTR with the inhibitor. Notably, while the c-MYC protein in HPB-ALL *MYC* T58A -5'UTR cells was little changed in response to hippuristanol treatment, c-MYC protein levels in both parental and HPB-ALL *MYC* T58A +5'UTR cells were markedly reduced in the presence of hippuristanol (Fig 3.13A). Consistent with this finding, HPB-ALL *MYC*

T58A -5'UTR cells were more resistant to growth inhibition induced by hippuristanol as compared to parental cells or the isogenic HPB-ALL *MYC* T58A +5'UTR (Fig 3.13B). These data suggest that the unwinding the structured 5'UTR of *MYC* mediated by the helicase activity of eIF4A is important for the translation of *MYC*. The fact that growth inhibition of hippuristanol is not fully rescued by expressing the exogenous c-MYC suggests that in addition to c-MYC, other targets were also regulated by eIF4A. Furthermore, we found that hippuristanol effectively inhibited the proliferation of a panel of human PTEN-deficient, c-MYC high T-ALL cell lines at relatively low concentrations (Fig 3.13C). These data collectively indicate the therapeutic potential of targeting eIF4A for T-ALLs dependent on pathologically activated c-MYC.

Figure 3.13 Hippuristanol Blocks the 5'UTR-Dependent Translation of c-MYC in T-ALL

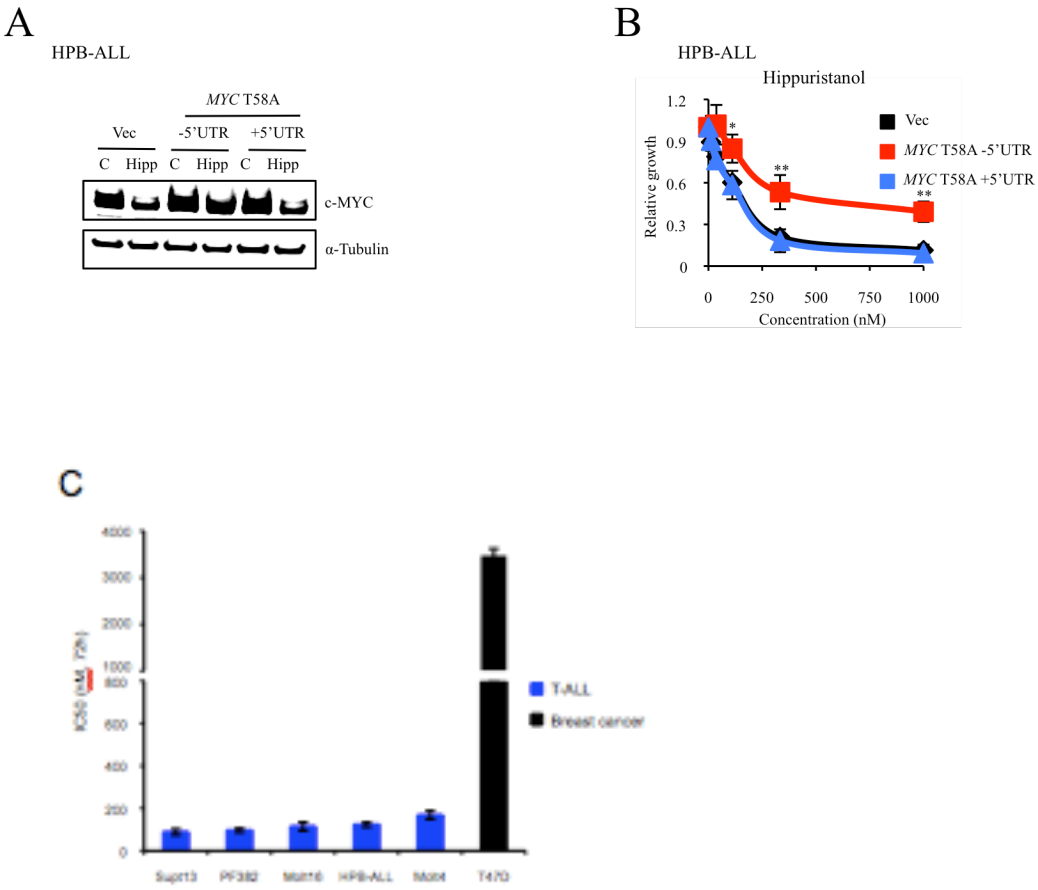
(A) The differential response of the c-MYC protein abundance in HPB-ALL cells expressing vector control, *MYC* T58A -5'UTR, or *MYC* T58A +5'UTR upon hippuristanol treatment. Cells were treated with 1μM hippuristanol for two hours before being subjected to Western blotting analysis.

(B) The differential response of the growth rate of HPB-ALL cells expressing vector control, *MYC* T58A -5'UTR, or *MYC* T58A +5'UTR upon hippuristanol treatment. Cells were treated with 1μM hippuristanol for seventy-two hours at indicated concentrations. DMSO treatment was used as negative control. **P*<0.05, ***P*<0.005 (n=3, Student's t test).

(C) Hippuristanol effectively inhibits cell proliferation of a panel of PTEN-deficient, c-MYC-high T-ALL cell lines with IC50 at nm range. Cells were seeded into 96-well plates and hippuristanol was applied at concentration gradients. Seventy-two hours after the drug treatment, cell proliferation was measured by MTS assay with IC50s calculated using Prism 6.

Contributions: I performed all the experiments for this figure.

Figure 3.13 (Continued)



To further investigate whether our finding in PTEN-deficient T-ALLs can be extended to other PTEN-deficient hematological malignancies, we screens another twenty-five hematological cancer cell lines and found that six of them showed low or undetectable PTEN levels (Fig 3. 14A). Impressively, c-Myc protein levels were inhibited upon either AKT or mTOR inhibitor treatment in all these six cell lines (Fig 3. 14B). We also confirmed that hippuristanol effectively inhibited c-MYC protein levels (Fig 3. 14C) and consequently blocked tumor cell proliferation (Fig 3. 14D) in these lines. These data

collectively indicate the efficiency to target the c-MYC translation regulatory pathway for c-MYC dependent hematological malignancies.

Figure 3. 14 Hippristanol inhibited c-MYC translation in hematological cancer cell lines.

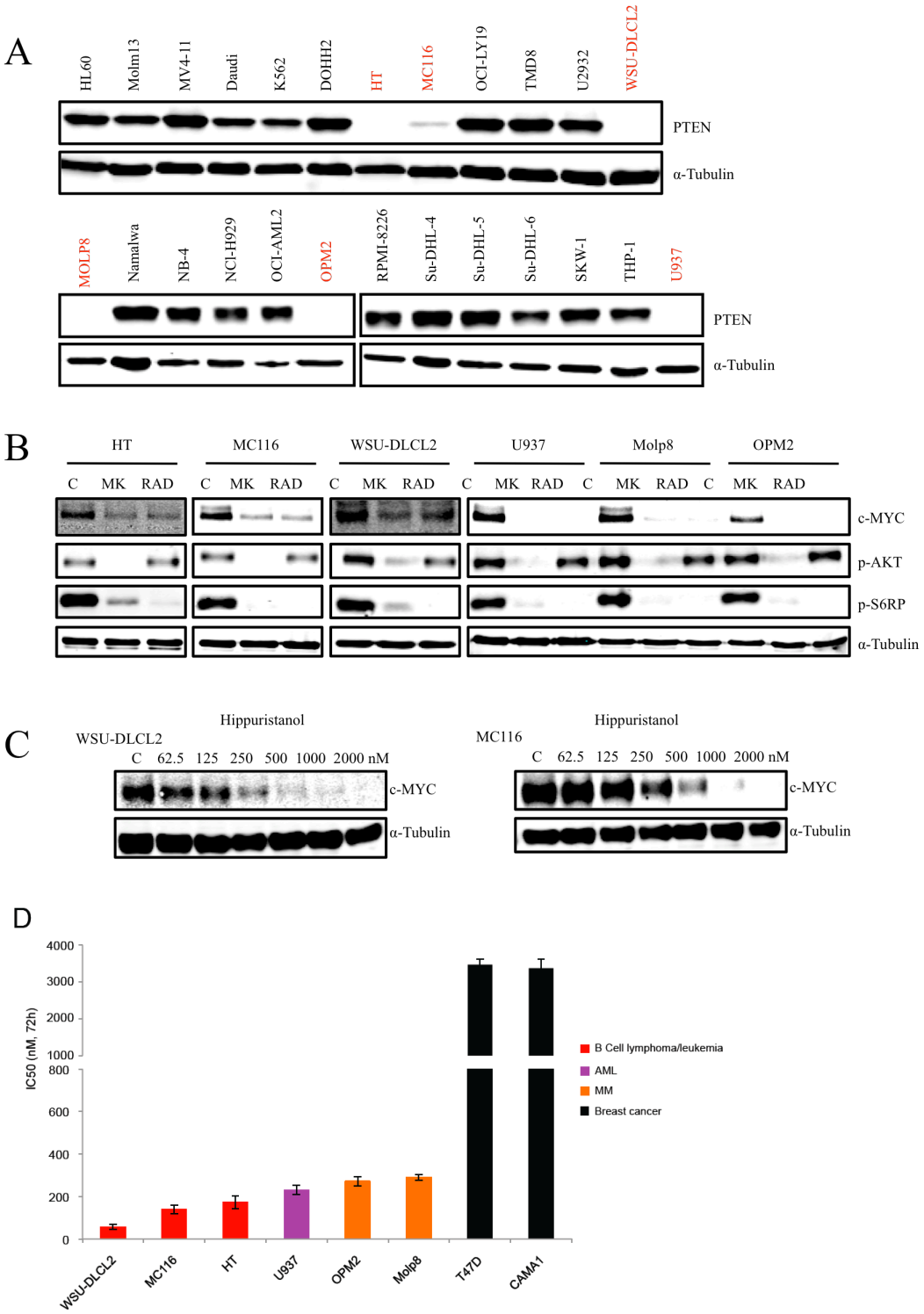
(A) Six (red) out of twenty-five hematological cancer cell lines (HL60, Molm13, MV4-11, OCI-AML2, THP-1, U937: AML; Daudi, Namalwa: Burkitt Lymphoma; K562: Chronic Myeloid Leukemia, CML; DOHH2, HT, OCI-LY19, TMD8, U2932, WSUDLCL2, Su-DHL-4, Su-DHL-5, Su-DHL-6: DLBCL; MC116, SKW-1: B cell lymphoma; Molp8, OPM2, NCI-H929, RPMI8226: MM; NB-4: Acute Promyelocytic Leukemia, APL) showed low or undetectable PTEN protein levels.

(B) MK2206 or RAD001 treatment inhibited c-MYC protein level in human PTEN null hematological cancer cell lines. 1μM MK2206 or 10 nM RAD001 was added for one hour and DMSO treatment was used as negative control.

(C) Dose dependent inhibition of c-MYC protein abundance in WSU-DLCL2 and MC116 cells after two hours treatment with hippuristanol.

(D) Hippuristanol inhibits cell proliferation in a panel of PTEN null, c-Myc dependent hematological cancer cell lines. Cells were seeded into 96-well plates and hippuristanol was applied at concentration gradients. Seventy-two hours after initial inhibitor application, cell proliferation was measured by MTS assay with IC50s calculated using Prism 6.

Figure 3.14 (Continued)



To test whether the inhibition of c-Myc translation is effective in c-Myc dependent cells that are insensitive to inhibition of c-Myc transcription, we tested Jurkat (T-ALL) and K562 (CML) cells. These lines both feature MYC amplification and are insensitive to JQ1 treatment (Zuber et al., 2011). To our interest, hippuristanol effectively inhibited cell proliferation in comparison to JQ1 in both cell lines (Fig 3.15A-3.15B). We further confirmed that JQ1 failed to inhibit c-Myc trancription in Jurkart cells (Fig 3.15C). On the other hand, hippuristanol effectively inhibited c-Myc translation, but not transcription or degradation (Fig 3.15D-3.15F). These data demonstrate the benefit to target c-Myc translation in hematological cancer cells insensitive to JQ1-mediated MYC transcription suppression.

Figure 3.15 Hippuristanol, a Small Molecule Inhibitor of elF4A, Blocks c-MYC Translation in JQ1 Insensitive Cell Lines

(A-B) The response of the growth rate of K562 cells **(A)** and Jurkat cells **(B)** upon hippuristanol or JQ1 treatment. Hippuristanol or JQ1 was applied to cells at the indicated concentrations for seventy-two hours before measurement.

(C) The expression levels of c-Myc mRNA in Jurkat cells remain unchanged upon 1μM JQ1 treatment at the indicated time points whereas JQ1 effectively inhibited c-MYC mRNA levels in JQ1 sensitive cell line OPM2. c-Myc mRNA levels were measured by quantitative PCR. GAPDH was used as control.

(D) The expression levels of c-Myc mRNA in Jurkat cells remain unchanged upon 1μM Hippuristanol treatment at the indicated time points. c-Myc mRNA levels were measured by quantitative PCR. GAPDH was used as control.

(E) The response of the abundance of c-Myc protein in Jurkat cells upon hippuristanol treatment. Hippuristanol was applied at 1μM at indicated times. DMSO treatment was used as negative control.

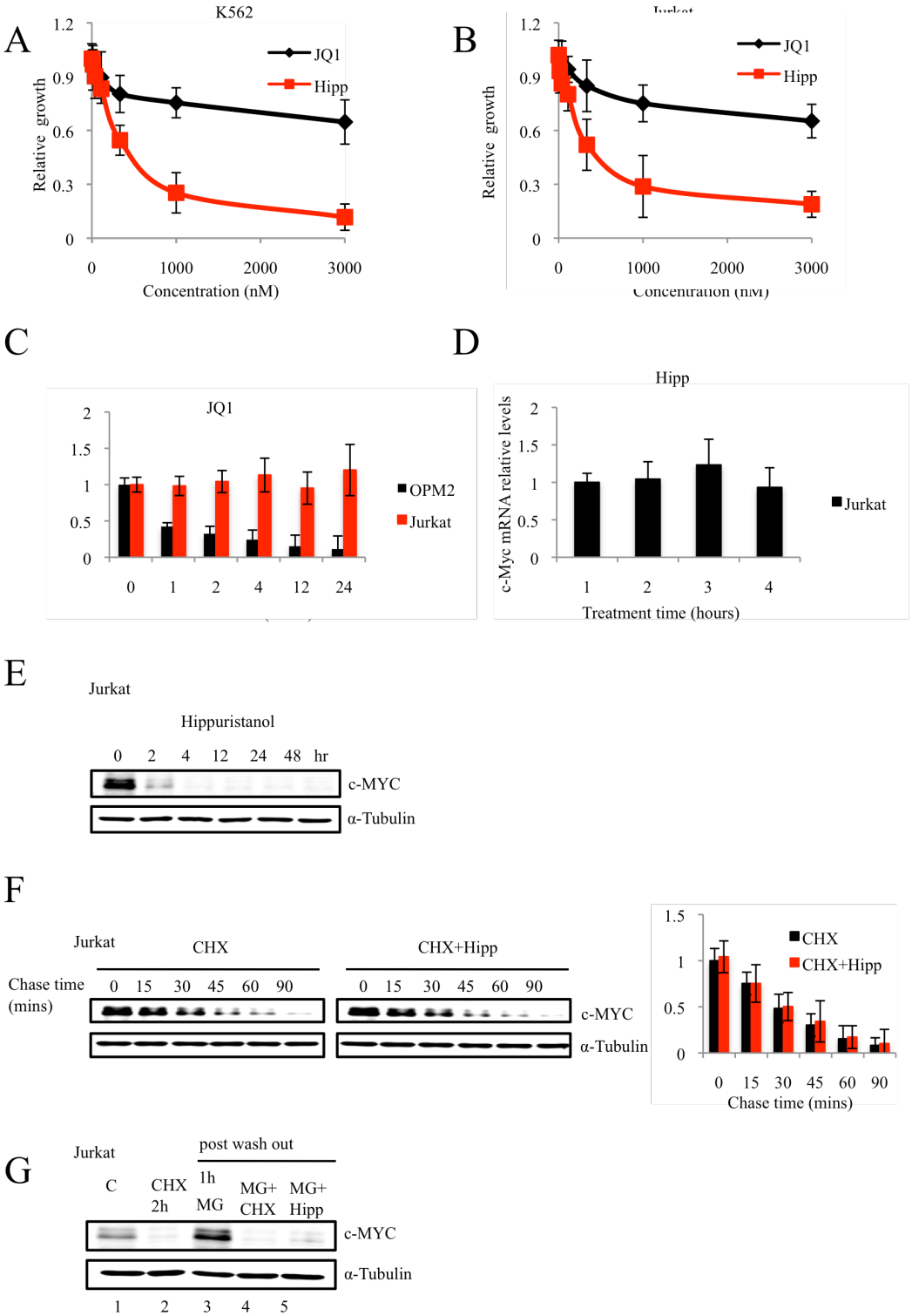
(F) Inhibition of eIF4A did not affect c-MYC protein stability. Jurkat cells were treated with 25μg/ml cycloheximide (CHX) with or without Hippuristanol for the indicated time points. Cell lysates were subjected to Western blotting, followed by quantification.

Figure 3.15 (Continued)

(G) The *de novo* synthesis of c-Myc protein in Jurkat cells is inhibited by hippuristanol treatment. Cells were pre-treated with 25μg/ml CHX for two hours to deplete c-Myc protein (lanes 1-2), and subsequently washed three times with PBS to allow new biosynthesis. MG132 was then added at 10μM either alone (lanes 3) or with 25μg/ml CHX (lanes 4) or with 1μM hippuristanol (lanes 5) for two hours.

Contributions: I performed all the experiments for this figure.

Figure 3.15 (Continued)



Given the fact that c-Myc is hyper-activated in various hematological cancer cell lines regardless of their PTEN status, we asked whether translational inhibition of c-Myc via eIF4A1 might also be utilized to target c-Myc in those cancer cells. Indeed, application of hippuristanol elicited robust inhibition of c-Myc protein levels and tumor cell proliferation in a panel of c-Myc dependent hematological cancer cell lines with normal expression of PTEN (Fig 3.16A-3.16D). We further confirmed that in these cells, the effect of hippuristanol is neither via transcription nor protein degradation but via translation (Fig 3. 16E-3. 16G).

Contributions: I performed all the experiments for this figure.

Figure 3.16 Hippuristanol, a Small Molecule Inhibitor of elF4A, Blocks c-MYC Translation in PTEN wt c-MYC Dependent Hematological Cancers

(A) Hippuristanol effectively inhibits cell proliferation of a panel of PTEN-wt, c-MYC-high T-ALL cell lines with IC50 at nm range. Cells were seeded into 96-well plates and hippuristanol was applied at concentration gradients. Seventy-two hours after the drug treatment, cell proliferation was measured by MTS assay with IC50s calculated using Prism 6.

(B-C) Dose-course **(B)** and time course **(C)** of c-MYC abundance inhibition in PTEN wt Burkitt lymphoma Daudi cells by hippuristanol.

(D) Hippuristanol effectively inhibits c-MYC protein abundance in a panel of PTEN wt c-MYC dependent hematological cancer cell lines.

(E) The expression levels of c-Myc mRNA in hematological cancer cells remain unchanged upon 1μM hippuristanol treatment at the indicated time points whereas. c-Myc mRNA levels were measured by quantitative PCR. GAPDH was used as control.

Figure 3.16 (Continued)

(F) The *de novo* synthesis of c-Myc protein in Daudi cells is inhibited by hippuristanol treatment. Cells were pre-treated with 25μg/ml CHX for two hours to deplete c-Myc protein (lanes 1-2), and subsequently washed three times with PBS to allow new biosynthesis. MG132 was then added at 10μM either alone (lanes 3) or with 25μg/ml CHX (lanes 4) or with 1μM hippuristanol (lanes 5) for two hours.

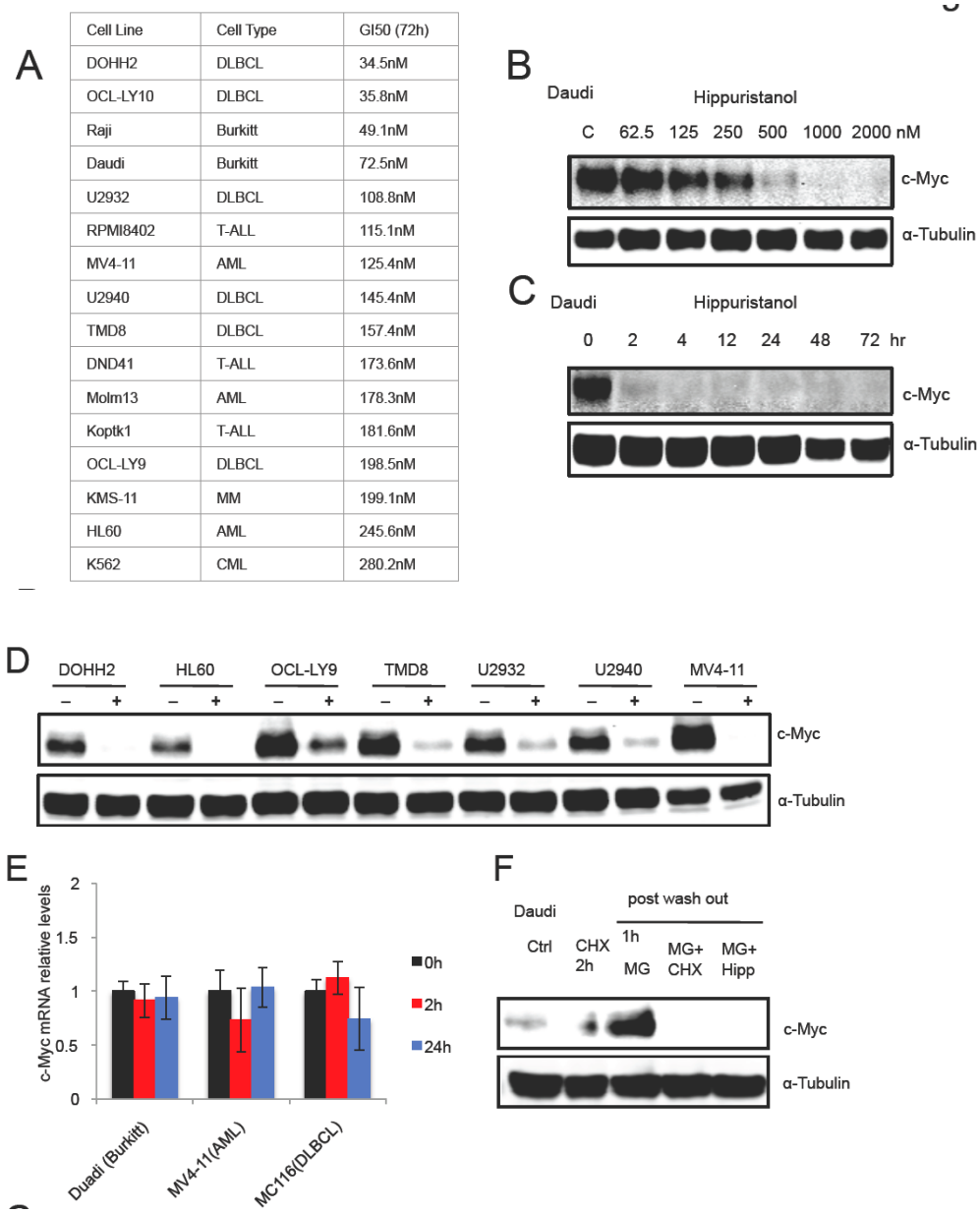
(G) Inhibition of eIF4A did not affect c-MYC protein stability. Daudi cells were treated with 25μg/ml cycloheximide (CHX) with or without Hippuristanol for the indicated time points. Cell lysates were subjected to Western blotting, followed by quantification.

Contributions: I performed all the experiments for this figure.

Figure 3.16 (Continued)

A

Cell Line	Cell Type	GI50 (72h)
DOHH2	DLBCL	34.5nM
OCL-LY10	DLBCL	35.8nM
Raji	Burkitt	49.1nM
Daudi	Burkitt	72.5nM
U2932	DLBCL	108.8nM
RPMI8402	T-ALL	115.1nM
MV4-11	AML	125.4nM
U2940	DLBCL	145.4nM
TMD8	DLBCL	157.4nM
DND41	T-ALL	173.6nM
Molm13	AML	178.3nM
Koptk1	T-ALL	181.6nM
OCL-LY9	DLBCL	198.5nM
KMS-11	MM	199.1nM
HL60	AML	245.6nM
K562	CML	280.2nM



Discussion and Future Directions

Most cases of T-ALL display upregulated expression of c-MYC, which frequently co-occurs with hyperactivation of the PI3K-AKT pathway, often through loss of PTEN (Gutierrez et al., 2009). However, the mechanistic link between the two events in this hematological malignancy has not been defined. Using a genetically engineered murine model of T-ALL driven by *Pten*-loss and featuring spontaneous overexpression of c-Myc, we found that the abundance of c-Myc protein in this model is critically dependent on hyper-activated PI3K-Akt signaling. More specifically, we found that the PI3K/mTORC1/S6K1 signaling axis robustly regulates the translation of *MYC* via a mechanism which requires the helicase activity of eIF4A to unwind the structured 5'UTR of *MYC* mRNA.

Our current results appear to be in contrast to previous findings from our labs and other groups that c-MYC overexpression renders cells resistant to PI3K/mTOR inhibition (Clegg et al., 2011; Ilic et al., 2011; Liu et al., 2011). For example, in a murine model of breast cancer conditionally expressing human $PIK3CA^{H1047R}$, a fraction of the tumors which recurred after $PIK3CA^{H1047R}$ inactivation had focal amplifications of *Myc*, which led to independence of oncogenic PIK3CA and resistance to PI3K inhibition (Liu et al., 2011). Notably, the focal *Myc* amplicon in these recurrent tumors identified by a high density SNP analysis lacked exon1 of *Myc* which encodes the *Myc* 5'UTR (Liu et al., 2011). Consistently, the c-Myc protein levels in these mammary tumor cells were not altered in response to PI3K and eIF4A1 inhibition. Together these findings indicate that

presence or absence of *MYC* 5'UTR may serve as a biomarker for predicting drug responses of c-MYC-dependent tumors to PI3K and eIF4A1 inhibition in human patients.

When activated, mTORC1 is known to regulate a number of translation initiation factors that are essential for translation, including eIF4E and eIF4B (Sonenberg and Hinnebusch, 2009). mTORC1 phoshporylates eIF4E-binding protein (4EBP1), an event that renders eIF4E available for binding to eIF4G and consequently, recognition of 5' cap by the eIF4F complex (Sonenberg and Hinnebusch, 2009; Zoncu et al., 2011). Another critical downstream factor of mTORC1 is S6K1. Upon activation, S6K1 phosphorylates eIF4B on Ser 422 to facilitate the translation initiation (Holz et al., 2005; Raught et al., 2004; Shahbazian et al., 2010; Shahbazian et al., 2006). eIF4B is a cofactor of the RNA helicase eIF4A which is known to be critical for unwinding structured 5'UTRs. It has been previously shown that eIF4A alone has low helicase activity but its activity can be significantly enhanced by binding to the phosphorylated eIF4B (Parsyan et al., 2011). In addition, S6K1 phosphorylates and destablizies PDCD4 (Dorrello et al., 2006), an eIF4A-binding protein that inhibits protein translation (Yang et al., 2003). The involvement of PDCD4 in our system remains to be determined. While both eIF4E and eIF4B have been previously implicated in the regulation of c-Myc (Moerke et al., 2007; Shahbazian et al., 2010), our study demonstrates a robust translational regulation of *MYC* through the PI3K-mTORC1-S6K1 signaling axis that activates the eIF4B/eIF4A initiation complex generally in hematologic malignancies dependent on c-MYC overexpression.

Aberrant transcriptional overexpression of c-MYC occurs in most cases of both human T-ALL and murine T-ALL models, often downstream of mutational activation of NOTCH1 in humans or *Myc* chromosomal rearrangements in mice (Guo et al., 2008; Guo et al., 2011; Liu et al., 2010; Weng et al., 2006; Zhang et al., 2011). However, the protein abundance of c-MYC is highly variable in T-ALL (Bonnet et al., 2011), suggesting that post-transcriptional regulation of c-MYC constitutes a major alternative mechanism of c-MYC activation in T-ALL. In line with this, previous studies have indicated that upregulation of *Myc* mRNA expression was not sufficient to induce lymphomagenesis (Hemann et al., 2005). It has been proposed that c-MYC protein abundance can be modulated by stability of c-MYC via phosphorylation status of T58 regulated by GSK3β (Bonnet et al., 2011; Gulati et al., 2008; Zhang et al., 2006). Also, c-MYC transcription has been proposed to be regulated by PI3K pathway and MAPK pathway via NFκB (Grumont et al., 2002). Here we show that, despite the presence of overexpressed *MYC* transcripts or *MYC* stabilizing mutations, as found in Burkitt lymphomas (Albert et al., 1994; Bhatia et al., 1993; Bhatia et al., 1994; Brennscheidt et al., 1994; Johnston et al., 1991; Yano et al., 1993), PTEN-PI3K dependent *de novo* synthesis of c-MYC protein is essential for sustaining the high abundance of c-MYC protein as well as for proliferation of these leukemia cells. Thus PTEN acts like a critical "gatekeeper" on this rate-limiting step in c-MYC biosynthesis.

Other than translational and post-translational control, c-MYC transcription also has been proposed to be regulated . For example, the above-mentioned post-transcriptional regulation of c-MYC by GSK3β and also regulation of c-MYC transcription by

Despite extensive efforts to target c-MYC for cancer therapy, direct inhibition of c-MYC protein has so far been unsuccessful (Darnell, 2002). Recent studies in hematological cancers have revealed a novel strategy of targeting c-MYC via suppression of its transcription through inactivation of BRD4 (Delmore et al., 2011; Mertz et al., 2011). These findings suggest that regulating *MYC* at transcriptional level or other levels may result in clinical benefit. Here we demonstrate that inhibition of c-MYC translation can also effectively suppress c-MYC protein synthesis and hematological tumor growth, providing novel avenues to target undruggable oncogenic lesions via suppression of their druggable regulators. Numerous pharmacological inhibitors targeting PI3K, AKT, mTORC1 or S6K1 are already in pre- and clinical development for cancer treatment (Liu et al., 2009b). A number of mTOR inhibitors, e.g. rapamycin and RAD001, have received FDA approval for clinical use (Lebwohl et al., 2013). Our study suggests that these drugs could be highly effective in treating T-ALL patients, an idea with the potential for a fast and meaningful translational outcome.

In addition, despite the promise of Bromodomain inhibitors to suppress c-MYC transcription in various cancer systems, the strategy inevitably has outliers and thus it is critical to identify additional potent therapeutical strategies for effectively targeting the c-MYC oncogene. Here we provide an effective alternative approach to specifically target c-MYC translation. It is intriguing to test whether JQ1 can also inhibit c-MYC in outlier cell lines which can not be inhibited by hippuristanol thus these two inhibitors can be used together to target a larger spectrum of human cancers.

Our study might also help explain the effect of the eIF4A inhibitor, hippuristanol, in other studies. It has been shown to inhibit the proliferation of HTLV-1-infected T-cell lines and ATL cells *in vitro* (Tsumuraya et al., 2011) as well as to reduce the viability of primary effusion lymphoma cells both *in vitro* and *in vivo* (Ishikawa et al., 2013). It was also reported to sensitize B cell malignancies to chemotherapy (Cencic et al., 2013). It is tempting to speculate these observed effects of hippuristanol might be mediated via the translational inhibition of c-MYC. It would be interesting to investigate whether other small molecules targeting eIF4A, such as pateamine A (Romo et al., 2000), silvestrol (Hwang et al., 2004) and the rocaglamides (Sadlish et al., 2013) can also inhibit c-MYC translation and whether targeting c-MYC translation through eIF4A1 is applicable to solid tumors as well.

Targeting translational components for cancer therapy has received less attention compared to other molecular targets critical for transcriptional regulation or post-translational modifications. One of the reasons might be the potential lack of selectivity of this approach (Robert and Pelletier, 2009). In the classic model of translational control by mTORC1, global translational regulation is achieved via phosphorylation of downstream factor 4EBP1, which binds to and inhibits the activity of eIF4E, a more general mechanism of translational control (Laplante and Sabatini, 2012). Our study suggests that certain oncoproteins, such as c-MYC, contain specific structured 5'UTRs and selective targeting may be achievable by inhibiting a less general factor, eIF4A, in translational control. We speculate that targeting eIF4A would likely be more specific

and less toxic, especially in tumors such as T-ALL where the *MYC* transcripts is greatly overexpressed.

The effective inhibition of PTEN-deficient hematological malignancies by suppression of eIF4A may also provide another therapeutic strategy to target PTEN-deficient tumors. PTEN is one of the most frequently deregulated tumor suppressors in various human cancers. Despite the large effort to target PTEN-deficient tumors using PI3K inhibitors, many PTEN-deficient tumors remain insensitive to PI3K inhibition or develop resistance after a short period of exposure (Ilic et al., 2011; Liu et al., 2011; O'Brien et al., 2010; Tenbaum et al., 2012). Our findings here demonstrate a promising alternative approach to target PTEN-deficient hematological malignancies. It would be interesting to test whether eIF4A suppression is effective in PI3K inhibitor insensitive or resistant PTEN-deficient hematological cancers.

The fact that hippuristanol is also effective to inhibit PTEN wide type c-MYC dependent hematological cancers also suggests that the mechanism of eIF4A regulated c-MYC translation is universal in hematological malignancies, regardless of their PTEN and PI3K signaling status. This could due to in PI3K signaling low cancer cell lines, some other upstream signaling such as the RAS signaling, RSK signaling and etc, upregulates the signaling to eIF4A. This also means that targeting eIF4A to inhibit c-MYC translation is universal in c-MYC dependent hematological malignancies.

Taken together, our study has shown a robust regulatory mechanism for the 5'UTR-dependent translation of *MYC* mRNA governed by the PI3K/mTORC1-S6K1 pathway and has provided conceptual evidence for testing eIF4A as a potential novel target in c-MYC dependent malignancy.

Experimental Procedures

Mice

LckCre (Hennet et al., 1995) and *Pten*$^{f/f}$ (Lesche et al., 2002) mice were crossed to generate experimental mice for this study. All animals were housed and treated in accordance with protocols approved by the Institutional Animal Care and Use Committees at Dana-Farber Cancer Institute and Harvard Medical School.

Cell lines and Culture

Hematological cancer cell lines were cultured in RPMI (Life Technologies) supplemented with 10% FBS (Life Technologies) and 1% Pen/Strep (Life Technologies). CAMA1 and T47D were cultured in DMEM (Life Technologies) supplemented with 10% FBS and 1% Pen/Strep. Primary cell lines were established from isolated thymocytes of moribund *LckCre*$^{+}$*/Pten*$^{f/f}$ mice and cultured in RPMI supplemented with 10% FBS and 1% Pen/Strep.

Chemicals

The following small molecule inhibitors were used in this study: BEZ235 (Maira et al., 2008), BKM120 (Elfiky et al., 2011), MK2206 (Chandarlapaty et al., 2011), RAD001 (Schuler et al., 1997) and hippuristanol (Bordeleau et al., 2006) were kindly provided by

Novartis Pharmaceuticals. The remaining chemical reagents were purchased from the indicated source: PF4708671 (Sigma), Lipofectamine 2000 (Sigma), MG132 (Sigma), Cycloheximide (Sigma), puromycin (Sigma), protease inhibitor tablets (Roche), phosphates inhibitor tablets (Roche).

Western Blotting

Cells were washed with cold PBS and then lysed in 1% Nonidet P-40 lysis buffer supplemented with protease and phosphatase inhibitors. 15μg of whole cell lysates were resolved by SDS-PAGE followed by transfer onto nitrocellulose membrane (Bio-Rad). Membranes were blocked and incubated with primary antibodies overnight. Immunofluorescently labeled anti-mouse IgG (Rockland Immunochemicals) and anti-rabbit IgG (Molecular Probes) were used to develop band intensities in the Odyssey system (Li-Cor). Quantification of band intensities was performed using Odyssey 3.0 software. The primary antibodies used for Western blotting in this study were purchased from Cell Signaling Technoloy, unless otherwise noted:, c-Myc, Pten, p-AKT (S473), p-S6RP (S235/236), eIF4A, eIF4B, Actin (Milipore), α-Tubulin (Sigma-Aldrich).

shRNA Constructs and Lenti-viral Infection

shRNAs constructs were purchased from the RNAi Consortium of the Broad Institute. Construct sequences are described in Supplemental Table 1. To produce lenti-viruses, 293T cells were co-transfected with shRNA constructs, Vsv-g and ΔR89 using

Lipofectamine 2000 at the ratio of 5:1:4. Viruses were collected on days 2 and 3 post transfection and one million cells were infected with 1ml virus. Forty-eight hours post infection, cells were selected with puromycin at 2ug/ml for two days and then maintained in puromycin at 2.5ug/ml.

De deno Synthesis Assay of c-MYC Protein

Cells were pre-treated with 25μg/ml CHX for two hours to deplete c-MYC protein and then washed with three times with PBS to allow new biosynthesis. MG132 was then added at 10μM either alone or with 25μg/ml CHX, or with inhibitors for one hour to two hours.

Retroviral Infections and FACS Sorting

MigR1-*MYC* T58A construct was a kind gift from Dr. Warren Pear (Abramson Family Cancer Research Institute). Human *MYC* 5'UTR sequence was obtained from NCBI and subcloned into MigR1-*MYC* T58A construct. Primer sequences are as follows: forward, 5'-ACCCCCGAGCTGTGCTGCTCG-3' and reverse 5'-CGTCGCGGGAGGCTGCTGGTT-3'. pCMV-PTEN was purchased from Addgene and subcloned into the MigR1 vector. With Lipofectamine 2000 (Life Technologies), retroviruses were generated by co-transfecting 293T cells with MigR1-based plasmids, Vsv-g and Gag at the ratio of 5:1:4. Viruses were collected on days 2 and 3 post-transfection and applied to cells. Seventy-two hours post-infection, GFP positive cells

were sorted using BD FACSAria IIu followed by inhibitor treatments or Western blotting analysis.

Histology, IHC, Wright-giemsa Staining and White Blood Cell Count

For histology analysis, primary tissues were isolated, fixed with 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin by the Dana Farber/Harvard Cancer Center Rodent Histopathology Core. For IHC, fixed sections were dewaxed and rehydrated, boiled in citrate buffer and then blocked. Antibodies were incubated overnight, and positive nuclei were detected by diaminobenziidine (Sigma). Sections were then counterstained with Mayer's hematoxylin. Antibodies used for IHC were Ki67 (Vector Laboratories), p-Akt (S473) (Cell Signaling) and p-S6RP (S235/S236) (Cell Signaling). For Wright-giemsa staining, cytospin slides (Thermo Scientific) with primary mouse bone marrow cells and peripheral blood smear slides were stained according to routine method (Lillie, 1944) using Wright-giemsa stain (Electron Microscopy Sciences). Mouse peripheral white blood cell counts were measured using HEMAVET Multispecies Hematology Analyzer (Drew Scientific).

Quantitative PCR

Total RNA was extracted from cells using TRIzol reagent (Life Technologies). cDNAs were reverse-transcribed using the SuperScript III first strand synthesis kit (Invitrogen) and subsequently utilized for quantitative PCR with SYBR Green Master Mix (Applied

Biosystems). Analysis was performed on triplicate PCR data for each biological duplicate and normalized to GAPDH. Primer sequences are as follows: MYC forward, 5'-CTACCCTCTCAACGACAGCA-3' and reverse 5'-AGAGCAGAGAATCCGAGGAC-3'; *Myc* forward, 5'-GTGCTGCATGAGGAGACACC-3' and reverse 5'-TTTGCCTCTTCTCCACAGACA-3'.

In vivo Xenograft and Inhibitor Administration

Five to six week old female C57 Bl/6 recipient mice were purchased from Jackson Laboratory. A total of two million thymocytes isolated from moribund $LckCre^{+}/Pten^{f/f}$ mice were injected retro-orbitally into sub-lethally irradiated recipients. For in vivo BEZ235 treatment, BEZ235 was dissolved into one volume of NMP (Sigma) and nine volumes of PEG300 (Fluka Analytical) and then gavaged at 35 mg/kg mouse daily (Maira et al., 2008), starting two weeks following transplantation. Studies were performed under the auspices of protocols approved by the Institutional Animal Care and Use Committees at Dana-Farber Cancer Institute and Harvard Medical School.

Cell Proliferation Assay

Hematological cancer cell lines were seeded onto 96-well plates at a density of 15,000 cells/well in 100 µl media. CAMA1 and T47D were seeded at 4,000 cells/well in 100 µl media and then drugs dissolved in DMSO or DMSO control was added. After seventy-two hours of incubation, cells were analyzed for cell viability by the addition of 20 µl

/well MTS reagent (Promega). After three hours incubation at 37°C, the signal from the viable cells was determined by measuring the absorbance at 490nM on a microplate spectrophotometer (Bio-Rad). IC50s were calculated using Prism6 (GraphPad).

Luciferase Assay

pGL3-+5'UTR was constructed via inserting the human *MYC* 5'UTR sequence into the pGL3-Basic (-5'UTR) (Promega) firefly luciferase reporter vector. Cells were co-transfected with pGL3 or pGL3-+5'UTR together with the pRL renilla luciferase reporter vector (Promega) as internal control at the ratio of 10:1. Forty-eight hours post transfection, inhibitors were added to cells for four hours and luciferase activities were measured with Dual Luciferase Reporter Assay System (Promega) and detected using Lumat LB 9507 (EG&G Berthold).

Statistics

Kaplan-Meier survival curves were constructed using Prism6 (GraphPad), and log-rank analysis was used to analyze the results. An unpaired, 2-tail student t-test was used to compute *P* values.

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CHAPTER FOUR

General Discussion

Summary

In this thesis, we studied the isoform dependence of PI3K catalytic subunits in PTEN null driven T-ALL and found that neither the p110α nor the p110β isoform is critical for its tumor maintenance. Study from other groups suggests that the p110δ and p110γ isoforms are important for tumorigenesis and tumor maintenance of PTEN null driven T-ALL. These studies together demonstrate the isoform specificity of PI3Ks in leukemia and provided conceptual evidence to develop PI3K isoform specific inhibitors for targeted therapy of T cell leukemias dependent on the oncogenic PI3K signaling. Given the potential toxicity of using pan-PI3K or AKT inhibitors, isoform specificity studies would provide evidence for more specific therapeutics.

Our study also utilized the T cell specific PTEN null mice with spontaneous c-MYC over-expression to study potential clinically relevant regulatory mechanism for c-MYC expression. Using this model, we discovered that c-MYC is a critical downstream effector of PI3K signaling in PTEN-deficient hematological malignancy. We found that the PI3K pathway controls c-MYC translation via mTORC1/S6K1. S6K1 regulates c-MYC 5'UTR through the translation initiation factor eIF4A, an RNA helicase critical of unwinding the structured 5'UTR of c-MYC for its translation initiation. Importantly, either knock-down or pharmacological suppression of eIF4A by its selective pharmacological inhibitor hippuristanol robustly inhibits c-MYC translation, consequently markedly reducing c-MYC protein abundance and blocking tumor cell growth. Our study has thus revealed a clinically relevant regulatory mechanism of c-

MYC translation and provides the rationale for investigating eIF4A as a therapeutic target for cancers dependent on c-MYC.

General Discussion and Future Directions

The results presented in this thesis demonstrated the significance to study isoform specific targeting of PI3K in T-ALL and also showed one novel approach to target c-MYC dependent hematological cancers via suppression of the PI3K signaling and the translation initiation factor eIF4A. These studies provided mechanistic evidence of how PI3K signaling induces oncogenic downstream c-MYC protein and how to block c-MYC translation for indirect targeting of the undruggable protein.

Several further experiments can be conducted to strengthen the biochemical links in this study. First, direct biochemical assays to show that eIF4A interacts with the 5'-UTR of Myc and whether the interaction is specific for c-Myc. One critical experiment to address this question is to do RNA-ChIP assay. Second, several helicases have by now been proposed to act on the 5'-UTR of Myc and it would be important to see that the helicase activity of eIF4A has a specific role to unwind the 5'-UTR of Myc. It is plausible to conduct in vitro assays showing that blocking helicase activity unloads Myc mRNA but not others from the polysomes. Third, to directly prove that PI3K signaling inhibits c-MYC translation, a protein pulse-labeling followed by immunoprecipitation experiment can be conducted. Alternatively, it would be informative to examine the distribution of c-Myc mRNA on polysome profiles under the same conditions. Fourthly, to prove that the

effect of hippuristanol is indeed on-target effect, mutants of eFI4A that can not be bound by hippuristanol can be used to show that the effect of c-MYC inhibition can be rescued. In addition, to demonstrate that the phosphorylation of eIF4B by S6K1 is critical for the signaling transduction from PI3K to eIF4A, phosphrylation mutant of eIF4B can be used to test whether the mutant can cause resistance to PI3K inhibition.

Further physiological experiments also could be conducted for preclinical and clinical indications. For example, to strengthen the therapeutical potential of using eIF4A inhibitors for clinical usage, in vivo effect of hippuristanol or eIF4A shRNAs can be tested. Also, to validate that the conclusion drawn from human cell lines and mice model is applicable to human patients, it is interesting to test the effect of PI3K signaling inhibitors and eIF4A inhibitor in human patients to check whether they can inhibit human patient primary tumor cell line proliferation as well as the c-MYC protein levels.

Another future direction is to check whether the presence or absence of c-MYC 5'UTR in hematological cancers is also a biomarker for the response to PI3K and eIF4A inhibitors. It is interesting to check whether the mutations in the c-MYC 5'UTR in several cancer cell lines, such as the Burkitt lymphoma cell lines, can cause resistance to eIF4A inhibition. If that is the case, it demonstrates that the MYC 5'UTR as well as mutations that are important for its function is a critical biomarker for response to PI3K and eIF4A inhibition.

One interesting observation is that the inhibition of eIF4E-eIF4G interaction by 4EGI-1

increased c-MYC protein levels in our system. This suggests that the blockage of c-MYC cap-dependent translation increased the efficiency of IRES translation, which has been suggested for c-MYC. Our preliminary data suggests that adding in the c-MYC 5'UTR into the luciferase construct significantly increased its transcription level, suggesting that IRES dependent translation is much more efficient than cap-dependent translation in this context. And this could be the reason why c-MYC protein level is dependent on eIF4A given that its activation is critical for unwinding the c-MYC 5'UTR and expose the IRES for more efficient translation machinery. Consistently, for solid tumors less dependent on the c-MYC oncogene such as breast cancer cell lines, the application of 4EGI-1 decreased c-MYC levels (Yi et al., 2013), suggesting that cap-dependent translation, the less efficient translational machinery, is the main translational machinery for c-MYC in c-MYC independent cancers.

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